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CYTOLOGY AND EVOLUTION

Cytology
and
Evolution

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10-7-61
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1960
ACADEMIC PRESS · NEW YORK · LONDON

ACADEMIC PRESS INC.
111 FIFTH AVENUE,
NEW YORK 3, NEW YORK

U.K. Edition, Published by
ACADEMIC PRESS INC. (LONDON) LTD.
17 OLD QUEEN STREET, LONDON, S.W.1

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4555

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Library of Congress Catalog Card No. 60-7864

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Printed in Great Britain by
WILLMER BROTHERS AND HARAM LIMITED
BIRKENHEAD

PREFACE

In some ways this is an unusual book. Unlike most text-books on cytology, it is much less concerned with the description of observed data than it is with their interpretation. An early training in zoology, a prolonged study of tissue culture with a temporary excursion into retinal physiology and colour vision, combined with a teaching career in histology and cytology have given the author an approach which is certainly unorthodox and may even be unique.

A description of the main classes of cells which appear in the outgrowths of simple tissue cultures is given in the first few chapters and this forms the starting point for a much wider discussion of certain aspects of cell physiology. A search is made for the origin and significance of these classes both in embryology and in phylogeny. It is then mainly the clues based on the phylogenetic evidence which are followed up, since these seem to be the more suggestive. They lead eventually to an investigation of certain versatile amoebae whose structure and behaviour are directly dependent upon fairly simple and universal factors in their environment. The possible influence of these same environmental factors, which are of such major importance to the amoeba, is then considered as a possible determinant for establishing the basic pattern for a simple colonial metazoan organism, and thus probably for the embryonic forms of the majority of invertebrates and, possibly, of vertebrates also. Emerging from this pattern, a tentative genealogical tree of the families of cells of an organism is then proposed and considered as a key to the manner in which the cells differentiate and to the manner in which they behave in tissue cultures.

The extraordinary importance of the immediate and local environment in the activities and differentiation of any cell is established in the first two parts of the book and the consequences form the basis for the rest. The theme is taken up in an analysis of the machinery which is at work in the various excretory and body-fluid-regulating systems in both invertebrates and vertebrates. By this machinery the tissue fluids in general and the immediate environment of the germ cells in particular are regulated in their own special ways. This study leads to several ideas on endocrinology which are likely to be very different from those current in most texts and which, though at this stage essentially hypothetical, may help in suggesting new methods of approach for further elucidation of this extremely complex field. Similarly, many features in the physiology and cytology of the retina are reinterpreted in the light of the observations and hypotheses developed in the earlier pages. Homoiostasis is again the dominating idea.

In the final chapter a method for approaching the study of cells is broadly outlined. Emphasis is laid on the necessity for considering both the manner in which patterns of cell-behaviour have evolved phylogenetically and also for an awareness of the way in which cells react to their own very special little environments and of how these are themselves altered by other cells in the vicinity and may be very different from that of the homoiostatically controlled main body-fluid, namely the blood.

In a sense, the book is a series of essays designed to re-orientate current ideas and thoughts. In no sense is it an exhaustive text. The information assembled and discussed is culled from a wide variety of fields, and, since he cannot be an expert in all those fields, the author is frequently conscious of treading either on thin ice or on other people's toes. Nevertheless the ideas which have been developed seem to be of some considerable importance to all those who are interested in the physiology of cells. Embryologists and endocrinologists in particular are likely to find much that is unorthodox, and even the neurophysiologist may need to question whether the micro-electrode is indeed the genuine Aladdin's lamp.

The author wishes to thank his immediate colleagues, Dr T. Vickers, Dr P. R. Lewis, Dr Mary Lobban, Miss Margaret Walker, and Mrs J. L. Pearson for stimulating discussions and for reading various parts of the manuscript. He also owes a special debt of gratitude to Dr Sydney Smith for reading the whole text and for his constructive comments and general encouragement. Both the author and the printers must be especially grateful to Miss Sylvia Elton for her patience in deciphering an almost illegible manuscript and converting it into a presentable typescript, and also to Miss Allison Howlett for her preparation of the illustrations.

Finally, the author wishes to acknowledge the kindness of all those authors and publishers who have allowed the use of illustrations from already published works, and, in particular, of those of his colleagues who have allowed the use of photographs before they were published elsewhere.

E. N. WILLMER

Cambridge, England
October, 1959

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INTRODUCTION

The conversion of the Victorian carriage into the racing car of today or of the 'Rocket'-type of locomotive into the 'Royal Scot' are object lessons in evolution. Step by step, some features have been modified and adapted; others have been discarded. Some entirely new characters have appeared, while certain basic structures have been maintained relatively unchanged throughout.

A knowledge of this history is not perhaps vital to the automobile or locomotive engineers of today, but it certainly helps the ordinary man to explain and understand the particular pattern which has evolved and it demonstrates the extreme changes which may occur by constant small 'adaptations'. Similarly a study of human embryonic development alone may be sufficient for the practising gynaecologist, but 'what do they know of London, who only London know?' A deeper understanding of the embryology of other animals may lead to better practice.

Step by step man has evolved from more primitive forms which are now for ever lost. So also have other animals. Nevertheless by combining a study of the evolution of a wide variety of animals, including man, with a study of their embryological development, certain basic patterns of the evolutionary process can be discerned; hypothetical, if not actual, 'common ancestors' begin to appear; and evolutionary history becomes more nearly a reality.

The basic units of the animal body are its cells, and, like the organs which they constitute and the animals to which they belong, they, too, have evolved. The evolution of animals is accepted and 'the outline of history' has been written; the evolution of the organs of the animal body is the basis of much zoological training; but the story of the evolution of the constituent cells and tissues is only beginning to be told. Cells are minute, and the methods of biochemistry, refined as many of them now appear to be, are, by contrast, gross. Fossils, which are so valuable in tracing the evolution of bones and organs, cannot help with cells. In recent years, however, the advent of new microscopical methods and the development of better physical and chemical techniques for studying cells in the living condition, and in their interactions with one another, have made the study of comparative cellular physiology a much more practicable and profitable pursuit and with its help some insight can be gained into the evolution of cells and tissues.

INTRODUCTION

The growth of cells *in vitro* by tissue culture methods has thrown into prominence, for the author at least, many important features of cell physiology. For this reason, some account will first be given of cells as they are seen under these conditions. This will be followed by a discussion of some of the problems which are raised when an attempt is made to relate the observations on cell behaviour in tissue culture to cells under embryological conditions and to the possible steps in the evolution of different types of cells. Finally some rather speculative pictures of the cytology of certain peculiarly complex and interrelated physiological systems will be painted on very wide canvases with the use of the rather impure pigments which will have been ground and mixed in the first two sections.

CHAPTER I

TISSUE CULTURE AND THE STUDY OF LIVING CELLS

In the animal body each organ is well known to be specially adapted for carrying out certain definite functions, and yet the whole animal is built to a precise plan characteristic of the species. In many ways, therefore, each species of animal must be considered as a somewhat special case and this specialization naturally entails particular problems for the physiologist. On the other hand, it is also well recognized that throughout the whole kingdom of vertebrates, for example, there is a common plan of organization, and that many of the general functions carried out by the different organs may remain much the same in all species; within this common plan, however, there may also be conspicuous differences in detail, from species to species, according to the special adaptations of the animal to its particular environment. These differences, unfortunately, sometimes tend to obscure the main pattern, for often, because of local conditions, human interest or some other essentially trivial cause, there is attached to them a much greater significance than they deserve in relation to the pattern as a whole.

The student of evolutionary cytology is therefore always up against the difficulty of deciding whether any particular structure or function has general significance, or whether it should be regarded as some aberrant specialization, produced by the organism in question to meet the peculiar demands of the environment, this last term being used in its widest sense. It need hardly be emphasized that the various tissues and structures in animals are often extremely complex and intricately organized, so that when the pattern of organization is only slightly modified there may be far-reaching secondary effects, and it is thus a matter of some difficulty to comprehend the nature of the primary modification and to separate it from the secondary results produced. At first sight, the adrenal medulla and a sympathetic ganglion do not appear as very similar structures, but, on further investigation of their embryological and phylogenetic development, the evolution of both from similar cells becomes obvious and the functions of both can be, at least partly, interpreted as variations on a common theme. The same might be said about the inner ear of man and the lateral-line organs of fishes, and about his thyroid gland and the endostyle of *Amphioxus*, and there are many other similar examples.

On the anatomical scale such homologues, among the vertebrates, as pectoral fins, forelegs, wings and arms are all, of course, now universally accepted; but on the cellular level there is still much that is mysterious.

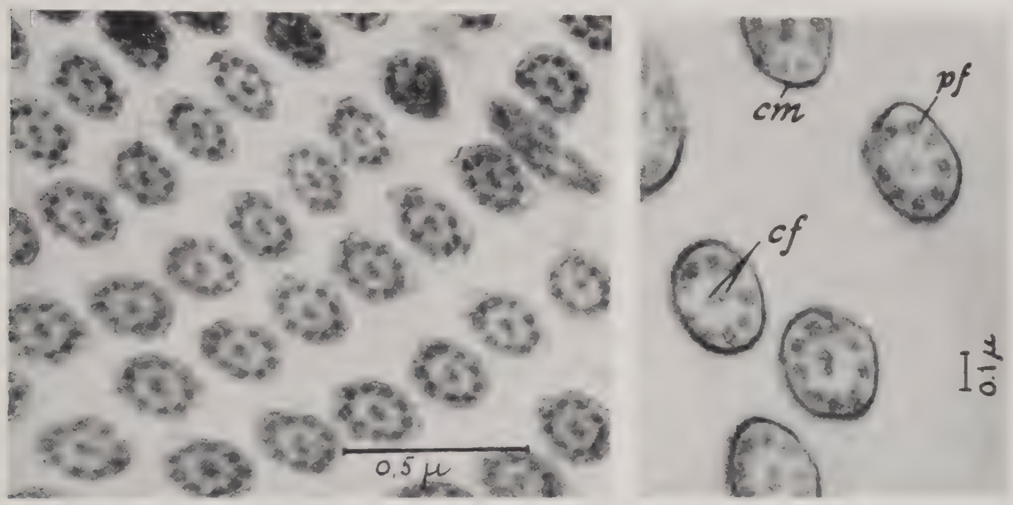
While the classical methods of histology and cytology, with their accent on cell structure and histological organization, can contribute much to the picture of the evolution of many of the various organs of the vertebrate body, they cannot reach beyond a certain point, because functional differences do not always involve parallel and visible structural or morphological differences. It is therefore obvious that the newer techniques which give information about histo- and cytochemistry and, especially, about cell behaviour will, in the future, have an ever-increasing contribution to make.

In the chapters which follow, the main accent will therefore be placed on those techniques and their results which allow cells to be investigated during activity, and a search will be made for those features of cell behaviour or their chemical activity which enable cells to be classified together as being similar on the one hand, or differentiated from each other in a fundamental manner on the other. Naturally, there is always the inherent difficulty of deciding what constitutes a fundamental manner and it is in elucidating this point that many of the real problems are raised. A few examples may illustrate the general standpoint which will be adopted.

Ciliated or flagellated cells occur in an extraordinary variety of situations in the bodies of animals belonging to nearly all the phyla. Yet, in all those animals which have been investigated except the Crustacea, the cilia and flagella, as seen by the electron-microscope and other optical techniques, seem to be built on a common plan, with variations of a rather secondary character (Fig. 1.1). They all have a core containing nine peripheral fibres, which are often double, and two central fibres. Is the conclusion to be drawn from this that there is only one possible type of flagellum, or is it that the essential flagellar pattern was developed early in evolution and has continued to be inherited as a useful potentiality by certain groups of cells, even if it is not actually called into play? Surely the latter is the more likely explanation, even if it does raise difficulties concerning the distribution of ciliated cells in different regions or parts of animals and with regard to the factors which cause their appearance or differentiation.

Similarly, muscular contraction and the presence in the cell of the proteins actin and myosin seem to be closely linked; are these proteins the only possible contractile elements? Or is their production again a primitive basic trait which has been handed on from one generation to another and from one animal to another to be produced, or not, according to circumstances and, when it is used, to be incorporated into special mechanisms adapted to serve particular ends?

The study of any one animal in isolation, whether it be man or amoeba, does not allow the wood to be seen for the trees. The specialization cannot be differentiated from the fundamental and common plan. Consequently it is necessary to try to extract the skeleton-key for the evolution of an organ or structure by studying features common to large numbers of organisms, and in studying the cells of that organ it may often be advantageous to rob them, if possible, of some of their higher flights of specialization so that their more fundamental basic pattern may emerge more clearly.



(*a*) Fig. 1.1 (*b*)

Electron-microscopic photographs of cross-sections of (*a*) Cilia from a mammalian renal tumour and (*b*) cross-sections of cilia from *Paramecium*, showing the typical pattern and arrangement of internal fibres. (Mannweiler and Bernhard, 1957; Sedar and Porter, 1955.)

Fortunately it seems likely, when tissues are grown by certain of the "tissue-culture" techniques, that the constituent cells almost immediately lose some of their morphological and physiological specializations, either temporarily or permanently, and such "simplified" cells may then be able to point to the road along which they have evolved. Admittedly, tissue-culture techniques are not without their specializing actions on cells, for cells have an extraordinary capacity for adapting themselves to their surroundings and, within limits, of adapting their surroundings to themselves. Indeed, evidence is accumulating from the study of "pure strains" of cells that prolonged life *in vitro* leads to many fundamental changes in cell activity, which can be profitably regarded as adaptations to the particular conditions of cell culture. Nevertheless, judicious comparison between the behaviour of cells in tissue culture, in embryological material, in pathological states, and their behaviour in corresponding or homologous tissues in a wide variety of organisms may often throw the main patterns of cell behaviour into relief. The actual meaning to be applied to the term homologous will have to be considered rather more closely at a later stage.

In bacteriological studies, it has, of course, become increasingly obvious in recent years that bacteria can often become adapted very quickly to life in surroundings which were at first unfavourable to them, even to the extent of producing new enzymes to deal with unfamiliar substrates. Whether this occurs by selection of favourable mutants or by some more directly adaptive mechanism is not always clear but the facts are undisputed, and there is very good evidence that enzymes can actually be produced *de novo* to deal with new substrates. How far similar adaptability is possible among the cells of metazoa is not yet so clear, but it must certainly be kept in the foreground as a possibility, and even a probability, when we are considering how cells could behave when the conditions of their life are altered. For example, there is now evidence that two clones of human cells, both originally derived from the same single cell, have developed quite different properties, one strain being highly malignant, the other not malignant at all (Sanford, Likely and Earle, 1954).

The critical study of cells in tissue-culture can thus, as indicated, provide a first approach to the study of the cells of the higher animals, and this approach is both illuminating and provocative. Some of the various methods used and the results obtainable with them must therefore be first reviewed in outline, for the behaviour of cells in culture depends very greatly on the method of culture employed. Each method imposes different conditions on the cells, and it is necessary to appreciate these effects in translating results from tissue-culture experiments to the behaviour of cells in the animal. Tissue culture, even if it does nothing else, succeeds admirably in emphasizing the plasticity and vital qualities of cells on the one hand, and in pointing towards the existence of a limited number of extremely characteristic and probably fundamental modes of cell behaviour on the other. It is this latter characteristic which is of peculiar interest in the study of evolutionary cytology.

THE METHOD OF TISSUE CULTURE

The development of the tissue culture method might have occurred long ago had it not had to wait for two main developments of technique and of knowledge in other fields. For all tissues from warm-blooded animals a satisfactory aseptic technique had first to be evolved. Tissues isolated in culture have only very limited defence against bacteria and moulds, so that either strict asepsis or the use of antibiotics is necessary and both are nowadays commonly employed. Secondly, the tissues of higher animals only survive and remain active in rather special media, so that much had first to be learned about balanced salt solutions, nutrients, growth stimulants etc., before any real progress could be made in the way of keeping tissues alive outside the body for any length of time. Although there had been earlier beginnings (Loeb, 1897, 1898), Ross Harrison (1907, 1910) is credited with the first successful tissue culture, when he obtained growth, of amphibian nerve fibres, *in vitro*, in a medium of clotted lymph. From these simple beginnings, progress was at first rapid, and numerous techniques, each with its own special application, were quickly developed. To the pioneering work of Carrel and his school at the Rockefeller Institute from about 1912 onwards are due many of the main methods of tissue culture (Carrel, 1912, 1913, 1923). Indeed, this school, together with that of Fischer (1930) in Copenhagen, produced much of the basic description of types of cell behaviour *in vitro*. In England, the division of cells *in vitro* was first described as a continuous process by Strangeways (1922) who, together with Canti (Strangeways and Canti, 1927; Canti, 1928), produced in 1927 a revolutionary, and now classical, cine-film which brought this process, and indeed many other essentials of cell behaviour, vividly before the eyes of the world. Much of the early work was carried out on embryo-chick tissues because these could be readily obtained in a very actively growing state, uncontaminated with bacteria, etc. The medium used for the growth of many of these pioneering cultures was a mixture of fowl blood-plasma and an extract of crushed chick embryo in a physiologically balanced saline solution containing Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, PO_4^{---}, HCO_3^- and glucose. Fowl plasma was used because, unlike that of mammals, it does not clot readily on standing, but produces a firm coagulum in the presence of tissue juices. The "embryo extract" contains both blood-coagulating and growth-promoting factors and can be readily obtained in a sterile condition. The most strict asepsis was preserved throughout the whole technique. This was many years ago and the position has now greatly changed. Since 1950, various synthetic media, in which all the constituents are of known chemical composition, have been successfully developed for certain strains of "normal" cells (Morgan, Morton and Parker, 1950; Evans *et al*, 1953; Healy, Fisher and Parker, 1954; Waymouth, 1955; Morgan, 1958); antibiotics are also extensively used nowadays to simplify the complicated techniques of asepsis, though there are certain limitations and possible dangers in their use. For example, the sulphonamide drugs, while relatively harmless to cells *in vitro* in comparison with their action on bacteria, do produce a reversible inhibition of cell division when used at concentrations of more than about 1:1,000. (Jacoby, Medawar and Willmer, 1941). Sulphanilamide itself is also known to poison the enzyme carbonic anhydrase.

Tissue culture today thus promises to become much simpler and to have more of its initially numerous variables under control. In consequence, more and more problems seem to be capable of solution by its use.

The primary aim of tissue culture is to maintain cells and tissues alive, active, differentiating, functioning, or growing under conditions in which the various processes can be observed directly or indirectly, measured and analysed in a situation uncomplicated by the presence of the interfering influences of other tissues in the body. Naturally the method can be applied to cytological, histological, embryological, biochemical and pathological problems and appropriate techniques have consequently been devised for particular purposes.

It is not necessary to describe the details of all the numerous techniques available; but some of the more important conditions of culture must be mentioned, so that the results obtained with them may be evaluated. The simplest technique is that known as the hanging-drop. In this, a small fragment of tissue (a few cu. mm. only) is placed in a drop of a physiologically balanced salt solution or nutrient medium, very often mixed with blood plasma, on a coverslip, and the whole is inverted over a hollow-ground slide and ringed with paraffin wax in order to maintain an air-tight seal. This, in essentials, was the original technique used by Harrison for the express purpose of finding out whether nerve processes grew out from nerve cells in the spinal cord of the frog, and by which he produced a major piece of evidence in support of the neurone theory of the central nervous system. By this method he was directly able to observe under the microscope how, after a few hours, the processes of the cells thrust themselves outwards as nerve fibres, by a sort of amoeboid movement, into the coagulated lymph which he used as medium (Fig. 1.2). Incidentally, the essentially amoeboid character of the terminals of the nerve processes which he then described is something which is perhaps insufficiently appreciated by neurologists and neurophysiologists of the present day as a potential property of nerve cells within the living body. Conditions *in vivo* and *in vitro* are, of course, very different, but it is unlikely that complete stability of the processes of nerve cells reigns in the body.

The hanging-drop method, with modifications, is eminently suited to cytological studies of short duration. Unless, however, the medium is renewed at frequent intervals or the tissues are transplanted to fresh medium, a limit of a few days only is set to their activity, by the scarcity of oxygen, high CO_2, lack of food, or the accumulation of toxic metabolites. In the early stages of such cultures, cells of various types may

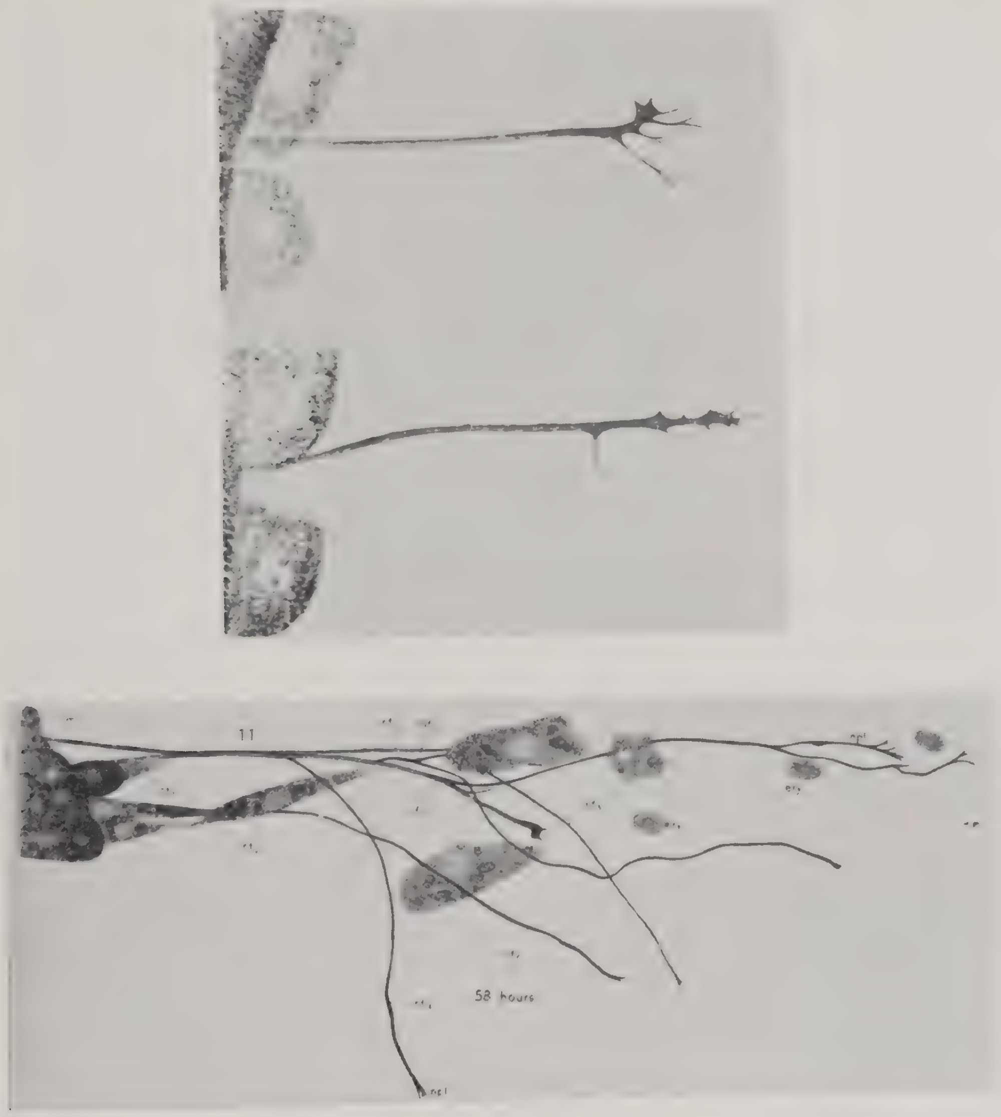


Fig. 1.2

The outgrowth of nerve fibres from the spinal cord of a frog in a medium of clotted lymph.

ms—central mass. nf.—nerve fibre. ct—connective tissue cell
ery—erythrocyte. npl—protoplasmic end of fibre (Harrison, 1907, 1910)

be persuaded by suitable media, temperature, etc., to emerge from the original piece of tissue (Fig. 1.3), especially if this is embryonic in character, or if more adult tissue has been previously exposed to the action of a dilute solution of trypsin (Simms and Stillman, 1937). On the whole, the younger the tissue the more actively do the cells emigrate and, for this reason, embryonic tissues have been most studied. The outgrowing cells mainly creep upon the surface of the glass or on the interphase between the medium and the air. Cells which become loose in the fluid tend to float away and get lost. If the medium is in the form of a gel, e.g. a plasma coagulum, then cells may also be able to invade the substance of the gel. The thinner the gel the more extensively is it invaded by the growing cells. Since, however, some cells liquefy the plasma coagulum, gels which are too thin sometimes break down, and thus there is a limit to the extent to which the blood plasma can be diluted for use as a supporting framework for the cells of each tissue.

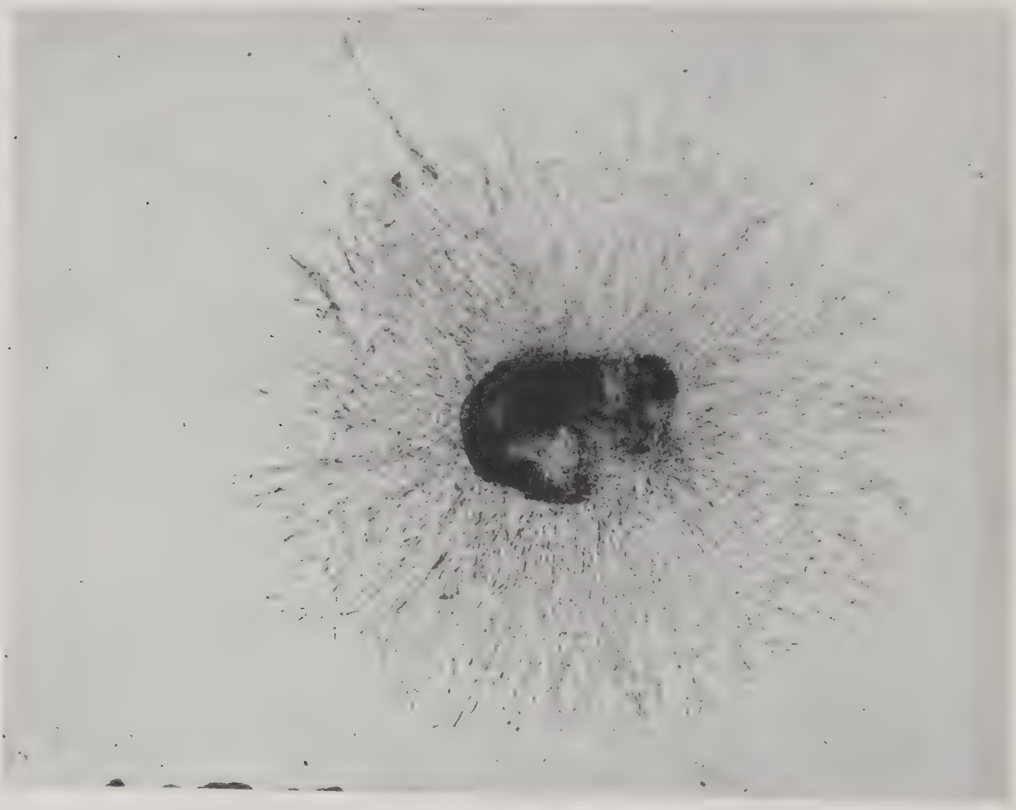


Fig. 1.3

Outgrowth of cells from a fragment of chick perichondrium in a medium of plasma and embryo extract. (Photograph by F. Jacoby).

In hanging-drop cultures the cells can be directly observed under the highest powers of the microscope, either with direct illumination or with such devices as polarised light, dark-field illumination, phase contrast, or other similar methods. They are also immediately available for experimental study, by microdissection, microinjection and most of the cytochemical techniques. Even more important, perhaps, is the fact that under these conditions the activities of the cells may be photographically recorded on cine-films, or directly on recording paper, either at normal speed, or at some higher speed when the processes to be observed are slow; the photography can also be combined with a stroboscopic device to record any rhythmic movements which may be occurring at too high a frequency for direct observation, as in the beating of cilia.

Although the cells under these conditions are ideally situated for study from the optical point of view, there is one major difficulty. The cells which migrate outwards from the central explant are not often immediately recognizable in appearance as normal constituents of the tissue explanted: in other words, they almost immediately change their shape and pattern as they leave their normal position. Thus, it must always be remembered that the cells which emerge are, in general, not physiologically, and certainly not morphologically, identical with the original cells of the tissue. Not only do the cells themselves change, but also the histological organization of the original tissue ceases to be maintained in the zone of outgrowing cells; it may, however, be so retained in the main mass of tissue, to a greater or less extent depending on the conditions of culture. The change in the outgrowing cells nearly always involves a simplification of their morphology. Moreover, the character of the change in the cells depends on the mechanical and physico-chemical properties of the medium into which they migrate; it depends also on whether the cells find a suitable surface upon which to cling, for when they become detached from the tissue into a fluid medium they immediately round up and remain relatively inert at the bottom of the drop, just as the white cells in the blood are spherical while they are in the circulation, but become more or less amoeboid when they make contact with a surface which they "wet" and to which they can adhere. This inertness of isolated cells may be only relative because it has now been found possible to maintain active growth in cells kept in a constantly agitated fluid medium where the cells are freely suspended (Earle, Schilling, Bryant and Evans, 1954, 1955; Owens, Gey and Gey, 1954).

Cells in culture, to some extent following the lines of least resistance, tend to flatten on to surfaces, to become spindly when enclosed in the substance of the coagulum, and to follow the pattern of any fibrous supporting framework which may be provided for them. In this connection it is worth while to contrast the local conditions of a columnar epithelial cell *in situ* with those which it meets when "growing" on the surface of a glass coverslip. *In situ* it has one surface resting on a "basement membrane" and bathed with tissue fluid. The opposite surface of the cell may be in contact with the fluid in the lumen of the duct. All other surfaces of the cell are adjacent to the surfaces of other similar cells. *In vitro* nearly half the cell surface is in contact with glass, nearly half is bathed in some abnormal but perhaps nutrient medium and the small remainder is in contact with adjacent but similarly abnormal cells. It is clear therefore that epithelial cells at least cannot be expected to function exactly as they do in the body when placed under these artificial conditions; deductions from cell behaviour in tissue cultures have therefore to be applied with the greatest caution to *in vivo* situations.

The Behaviour of Cells in Culture

The simple methods of tissue culture just described immediately and dramatically emphasize several points which are essential to the appreciation of cell behaviour as a whole. The cells of a tissue react almost immediately to changed conditions; many respond not only biochemically but also by increased movement and by changes of shape and form, which in tissue cultures are often in the direction of greater simplification with loss of morphological characteristics. The movement of the cells, in most cases, eventually leads to a thinning out of the explant and to a more uniform spacing of the cells on the available surface, so that each cell gets a suitable living-space with sufficient supplies of oxygen, etc. Indeed, in cultures obtained either by first dissociating tissues with Ca^{++}- and Mg^{++}-free trypsin solutions and then making suspensions of the separated cells and allowing them to settle on the glass surface of the culture vessel (Moscona, 1952; Rinaldini, 1954) or by some similar technique, it has been shown that a given size of vessel, as with bacteria, can only support a limited number of cells, independently of the amount of nutrient medium in the vessel (Earle, Sanford, Evans, Waltz and Shannon, 1951). Necrosis sets in when this number is exceeded.

The rate and direction of movement of cells is always partly determined by the presence of neighbouring cells which may be of similar or of different types, and there are many other factors, some intrinsic in the actual tissue itself and others dependent on the environment, which determine the activity of the cells in a culture.

Among external factors, the orientation of any micellar particles in the medium is of great importance. For example, if a fragment of embryonic heart muscle is placed at the centre of a plasma droplet suspended within the space of a minute triangle of glass rod, the tissue first of all brings about the coagulation of the plasma and then its cells begin to emerge into the plasma. They do not, however, emerge evenly, but they come out in greater numbers and proceed more quickly in directions at right angles to the glass sides of the triangle (Figs. 1.4, 1.5). The reason for this is that, during and after the formation of the clot, there is some contraction of the gel and this produces uneven stresses in the whole coagulum (Weiss, 1929). It is these lines of force which appear to guide the movement of the cells, probably not directly but because

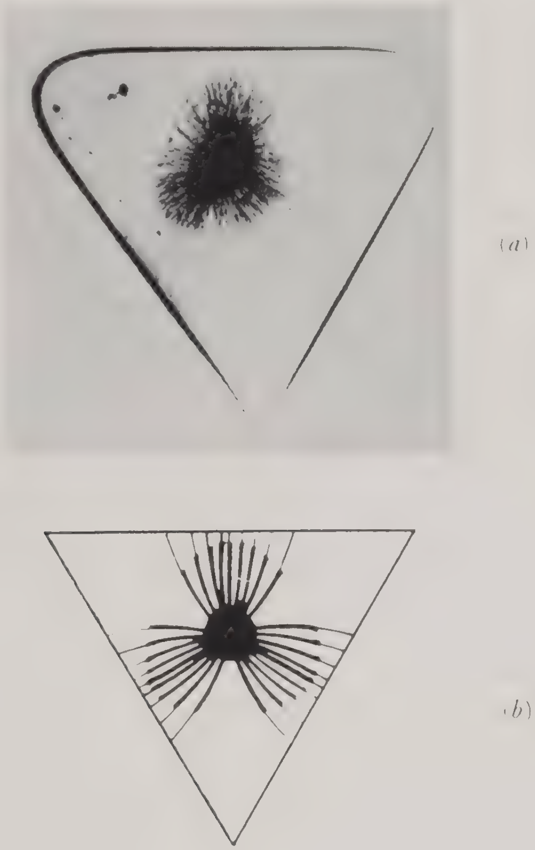


FIG. 1.4

(a) Cells emerging from a tissue culture suspended in a plasma clot contained within a triangular frame. Note the tendency for the cells to orientate themselves towards the sides, rather than towards the angles, of the triangle. (b) Diagram indicating lines of tension. (Weiss, 1929.)

the fibrous protein particles in the gel have become orientated during the contraction. In some way these particles induce the cells to elongate in a direction parallel to their own long axes. A similar result is obtained by culturing cells on the scales of certain fishes when it is found that the cells align themselves along the grooves in the scales (Figs. 1.6, 1.7) (Weiss, 1958).

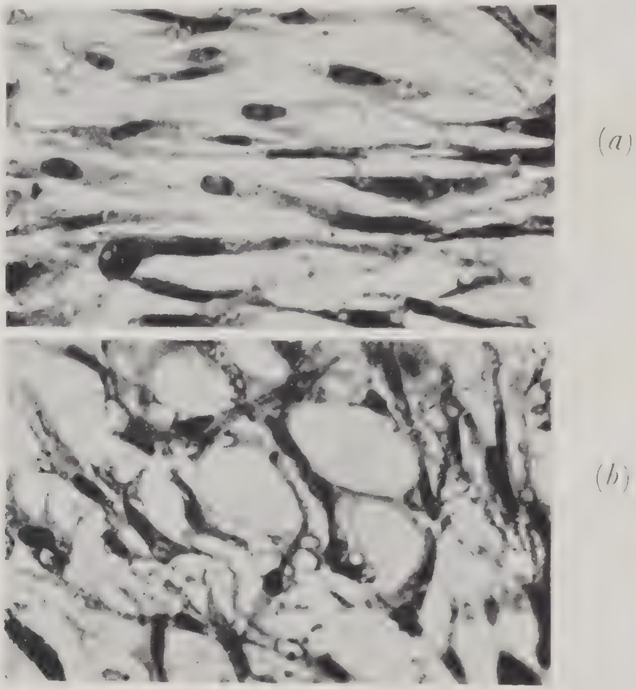


Fig. 1.5
High-power photographs of the cells in the culture shown in Fig. 1.4a. (*a*) cells facing the sides of the triangle. (*b*) cells facing the angles of the triangle. (Weiss, 1929.)

Not only can the cells be orientated in this way, but their shapes may be similarly influenced; cells tend to be more spindle-shaped when they are under tension or lying on an orientated surface. It is notable that cells in a coagulum are always more truly spindle-shaped (i.e. in three dimensions) than when growing on a surface. The microscope, which gives a sharp image only of a single optical plane, thus easily leads to false interpretations of the size and shape of cells unless the conditions are fully appreciated "in the solid". The spindle-shaped cells often appear much smaller than those flattened on the surface, though in fact their volumes are presumably similar.

The first and vitally important point, therefore, which tissue culture in this simple form emphasizes, is the lability of cell morphology in response to changed conditions, and this property of cells is seldom fully appreciated by histologists and cytologists accustomed only to the study of fixed and stained material. It may perhaps be argued that conditions in tissue culture are very different from those in the body and, consequently, that they are in some ways irrelevant to normal cell physiology: in general, this is certainly true. Nevertheless the conditions in a wound, for example, or in the culture chambers which have been used for the observation of cells *in situ* in different situations in the animal body, as in the anterior chamber of the eye, in the "windows" sometimes used in the ears of rabbits (Sandison, 1928; Clark and Clark, 1932; Ebert, Sanders and Florey, 1940) or in the skin-flaps on the backs of mice (Algire, 1943, 1949) are not actually very different from those in a tissue culture, and it is generally assumed that the cells in these situations are doing very much what they normally do, or can do, under conditions which can be truthfully described as "physiological". The main and most constant difference between the environment of cells in culture and that of cells in the body, probably lies in the presence or absence of a circulating blood supply, and of the cells which come with it. Other factors may, of course, also arise to modify the

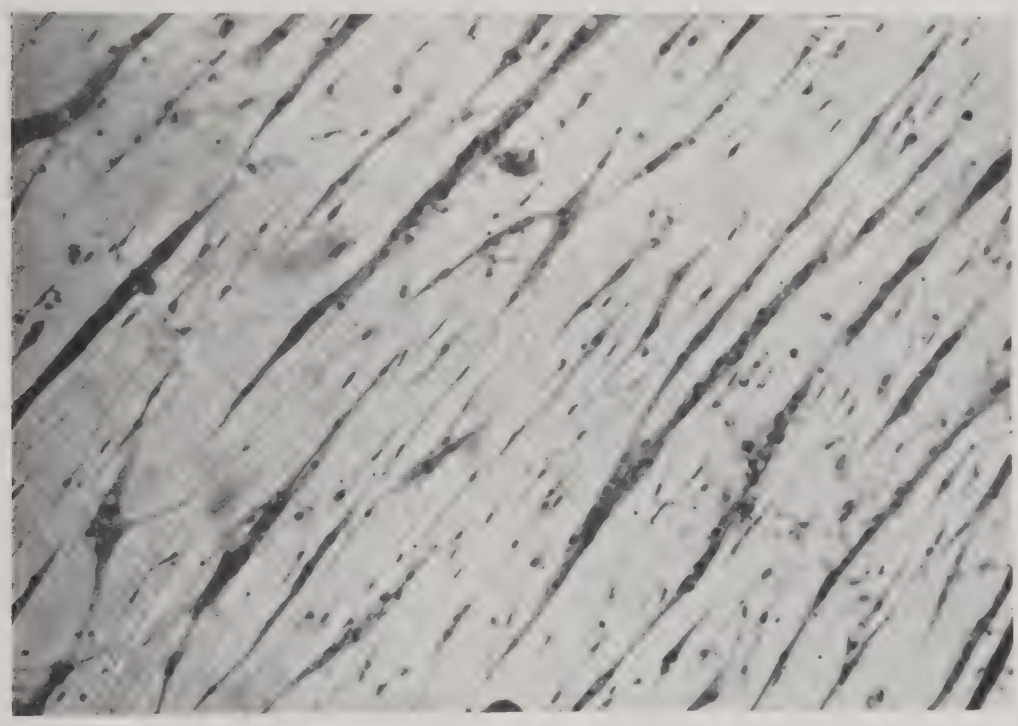


FIG. 1.6

Cells of a tissue culture orientated along the grooves of a fish-scale.
(Photograph by P. Weiss.)

immediate surroundings of the cells, but these depend very much on the method of culture and are sometimes important and sometimes not.

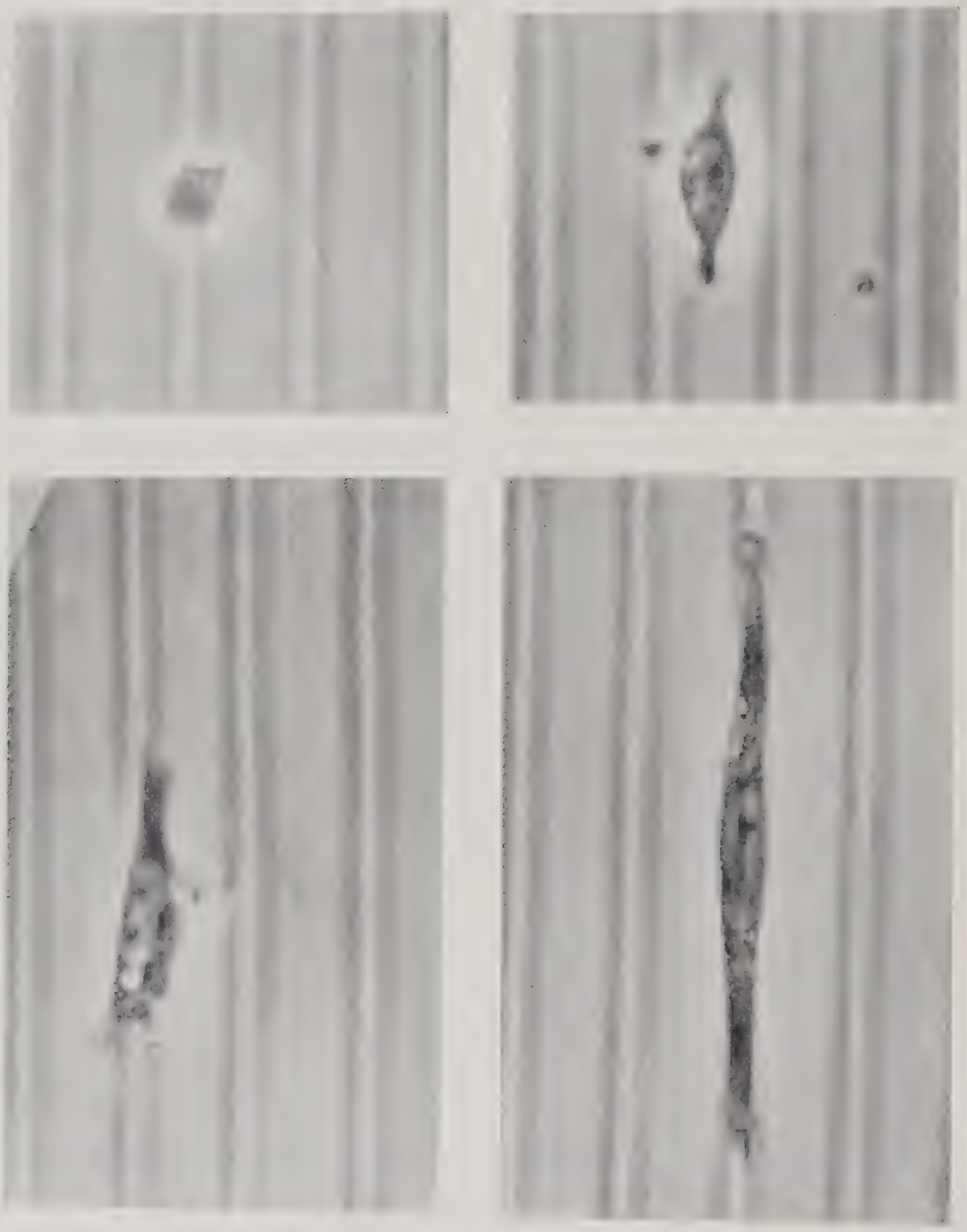


Fig. 1. 7
Four stages in the orientation of a cell along the groove of a fish scale.

(Photographs by P. Weiss.)

The second point which naturally emerges from the study of cultures involving the use of tissue explants, concerns the relationship between the cells which emigrate from the explant and those which were present in the original tissue when it was explanted. What are the changes, other than those of purely morphological character and those dependent on the mechanical conditions reigning in the culture which occur in the emigrating cells, and how far are the cells of the outgrowth different from those in their original situations? This is of such fundamental importance that it will form the subject-matter of the succeeding chapters and provide a starting point for a study of evolutionary cytology.

Nearly everything which has been said in this chapter about tissue culture has had direct reference to the hanging-drop method. In cultures in Carrel flasks, in roller tubes and made by all those methods whereby the cells are encouraged to emigrate into the medium (Parker, 1958, Willmer, 1958), the same considerations concerning cell morphology and function apply, and they apply with even greater force to the more recent techniques in which cells are first obtained in suspension and are then grown under conditions more akin to those in which bacteria are usually cultured. On the other hand, in the methods of "organ culture" in which pieces of tissue are cultured in such a way as to restrict outgrowth, and in which attention is concentrated on preserving functional activity within the explant, the cells may remain very much as they appear *in situ* in the body and their functional activities are probably much more akin to those in which they are normally involved. For many purposes, this type of culture is ideal and observations dependent upon its use will be freely quoted, but for continuous studies of cell behaviour it is not so convenient as those other methods where the comparatively isolated cells can be kept under observation almost continuously. Broadly speaking, "organ cultures" are used for investigations of tissue function and for studies of organized growth. For unorganized growth and cytological studies, other methods are at present more suitable, but the results always require interpretation on the lines which are under discussion.

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Chapter 2

CELLS IN TISSUE CULTURES: MECHANOCYTES

The study of cells emigrating during the early stages of growth from cultures of embryonic or adult tissues from a wide variety of vertebrates including, for example, fish, amphibia, birds, mammals and man, reveals the interesting and perhaps surprising phenomenon that the resulting morphological appearance of the cells, although not always identical, generally conforms to one of a very limited number of patterns. In other words, the cells which emerge are neither all immediately reduced to one tissue-culture type nor do they maintain the great diversity of types to be found in the original explants. The emergent cells can, in general, be grouped into three or four main categories according to their appearance, type of movement, staining reactions, and general growth-habits and requirements. These main categories are: epitheliocytes, mechanocytes (fibroblasts) and amoebocytes (wandering cells). Nerve cells form a further category with rather different characteristics and will need to be considered separately, and so too will one or two other smaller groups of cells.

Each tissue or organ, from whatever animal it is obtained, produces a pattern of growth which, while not specific to that organ or tissue alone, is characteristic of it. The significance of these major patterns is obviously, in itself, a point of some interest, but the interest must inevitably deepen when it is realized that the patterns are not only skin-deep and morphological, but also reflect the existence of underlying differences in physiology and behaviour among the cells present.

Although, on the basis of what we have already discussed in relation to the lability of cell form, too much weight must obviously not be placed upon the outward appearance of cells, for purposes of diagnosis, the existence of only a few varieties of cell-form as observed in hanging-drop cultures will be seen to argue in favour of the existence of a correspondingly limited number of standard types of cells which do not readily transform from one into the other; and it will be shown also that of these main structural patterns each reflects a different behaviour. Moreover, these differences in behaviour are not generally connected in any causal manner with the different conditions in which the cells find themselves in the culture. It is as though the cells which emerge in hanging-drop cultures either belong to, or revert to, only a few types which then continue to reproduce their own kind. It is our immediate problem, therefore, to enquire into the nature and properties of these types, for they cannot be accidental, nor can they only be the outcome of the environment in the tissue culture, for it frequently happens that two or more types of cell exist simultaneously in one and the same culture (Figs. 2.1, 2.2). Some features in the morphology of the cells in tissue culture may certainly be the direct effect of environmental conditions but the cell types under discussion are, equally certainly, not produced in this way. It must be remembered also that the same main types of cells have emerged, so far as tests have been applied, in tissue cultures from fish, amphibia, reptiles, birds, mammals

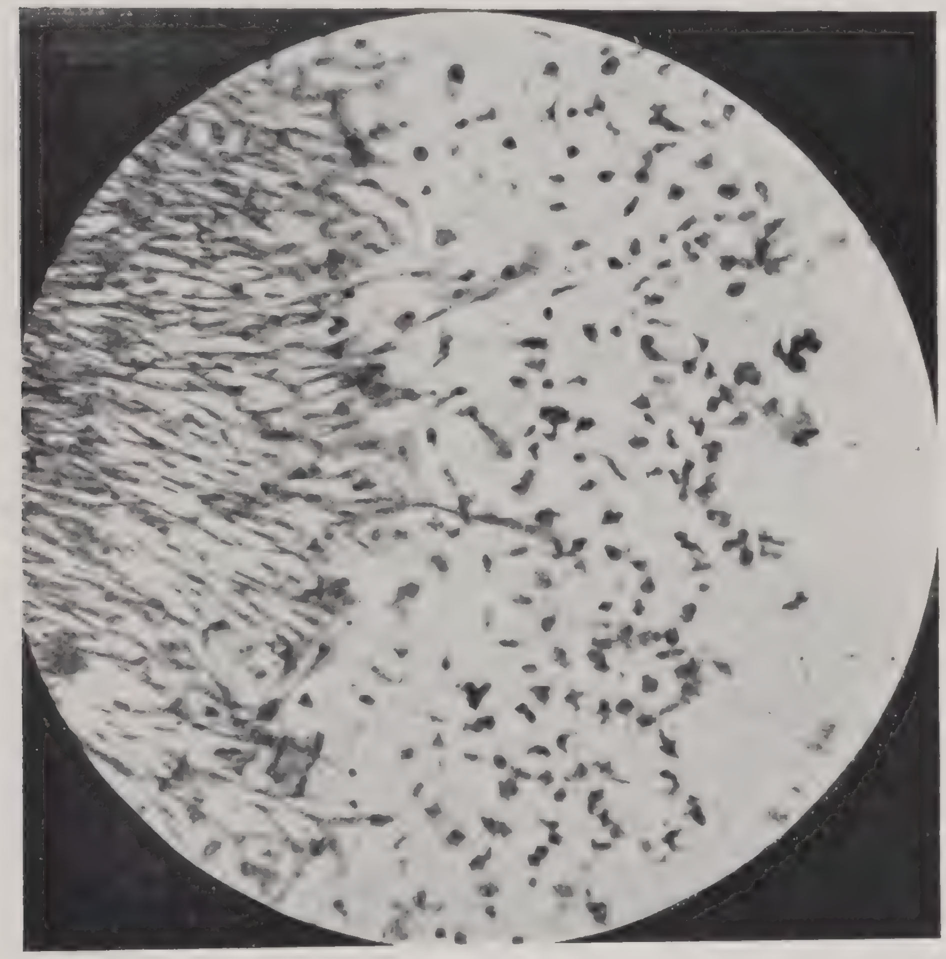


FIG. 2.1

The outgrowth from a culture of chick heart in which mechanocytes (fibroblasts) are seen on the left and wandering cells appear on the right. (Fischer, 1930.)

and man. This presumably implies some rather basic and primitive grouping of cells into these categories of behaviour.

The first questions to be answered are whether these cells which emerge from the explants actually exist as such in the tissues when they are explanted and belong only to those classes whose cells wander out, or whether they arise by a morphological simplification of cells of various sorts and kinds by a process which might be described as a histological dedifferentiation, or even as a true functional dedifferentiation, i.e. a reversion to a more embryonic form. Fortunately, hanging-drop cultures, backed by other techniques, readily provide the answers; and it will be profitable to examine the behaviour of tissues from various organs in relation to the cells which emerge from them in such cultures.

Cultures from the Chick Heart

From the early days of tissue culture the heart of the chick embryo has been used as a starting point for numerous studies and it will again form a very good introductory tissue. At the age of about seven days the heart of the embryonic chick is, of course well established as a contractile organ. It contains, in addition to the blood cells of various types within its cavities, differentiated muscle cells, connective tissue cells, endothelial cells and macrophages.

In a hanging-drop culture in fluid medium, e.g. a physiologically balanced salt solution like Tyrode's solution, serum, extract of embryo tissue or a mixture of these, the majority of the cells which emigrate have a morphology somewhat similar to that indicated in Fig. 2.3. In detail, the appearance of the cells may be recognizably different in the different media but the general character remains unchanged. The occurrence of such things as fat droplets, granules and vacuoles in the cytoplasm is always very dependent on the medium. For example, cells in dilute serum are usually clear and hyaline while in embryo juice they become more or less charged with fat droplets. The cells, as seen in speeded-up cine-films migrate more or less radially outwards, with a peculiarly characteristic gliding movement, on the surface of the glass or of the drop. The movement of each cell is thought to be brought about by waves of constriction starting anteriorly in the surface layer of the cell and passing backwards in such a way as to press the more fluid contents of the cell forwards (Lewis, 1940). Somewhat pointed pseudopodial processes are formed, mostly at the anterior end of the cell, i.e. in the direction of movement. In the somewhat similar type of movement characteristic of blood lymphocytes, when they are creeping in the "hand-mirror" form on a suitable glass surface, i.e. with a broad anterior pseudopodial region and a narrow and constricted posterior region or "uroid", the constriction band has been observed to be stationary and the cytoplasm appears to flow through it (de Bruyn, 1946; Lewis, 1931). Whether this is also true of these cells emigrating from the heart is not yet certain. While the cells from the heart move individually and separately, they are influenced by others in their neighbourhood and are slowed down by contact with each other (Abercrombie and Heaysman, 1952). The whole pattern of growth assumes the form of a network of clear cells, each somewhat fan-shaped in the periphery of the outgrowth and inclining more to be spindle-shaped nearer the original tissue, where they are densely packed

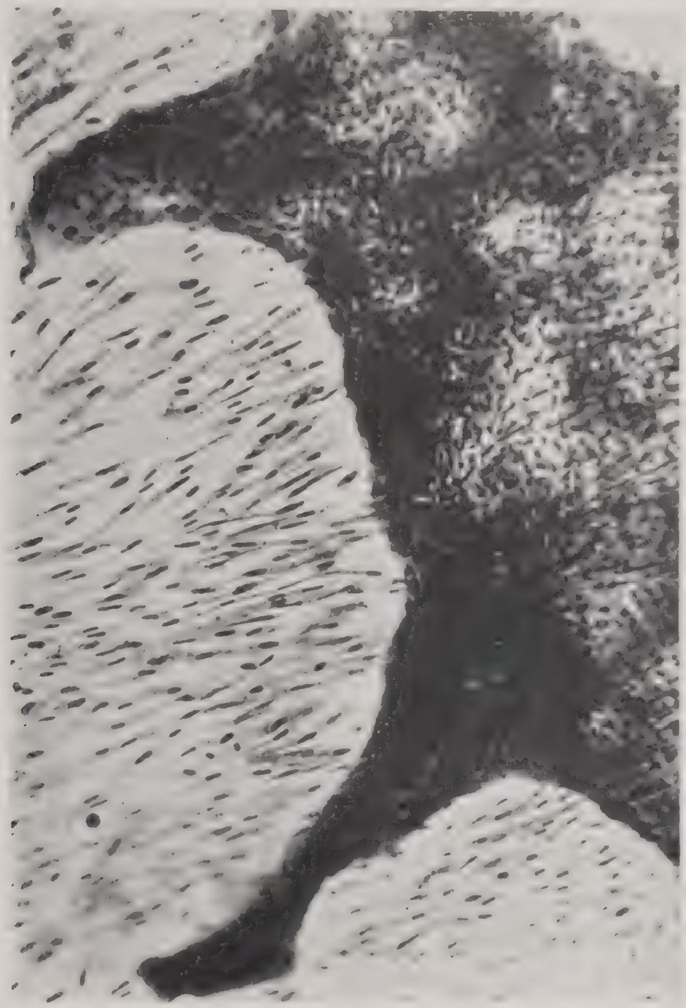


FIG. 2.2

A mixed culture in which epithelial cells are seen on the right and mechanocytes (fibroblasts) are seen on the left. (Fischer, 1930.)

together. The cells mostly have a single nucleus with two nucleoli, though the number of nucleoli seems to vary, from one to three or four, in a manner which is as yet unexplained. The cytoplasm contains numerous rod-shaped or filamentous mitochondria which move about independently within the cell and can sometimes be seen to fragment. The cytoplasm may also contain fat droplets, a few granules, and vacuoles which increase in number when the cells are short of sugar and which stain with neutral red (Lewis, 1922). If presented with acid vital dyes, like trypan blue, these cells, by comparison with certain others, e.g. macrophages, only take up a very small quantity, and that rather slowly. These cells are generally known as "fibroblasts" though, for reasons which will be discussed later, the term "mechanocyte" is preferable. The term "fibroblast" already has a more restricted use in connexion with those cells in the body which actually form the fibres of connective tissue.

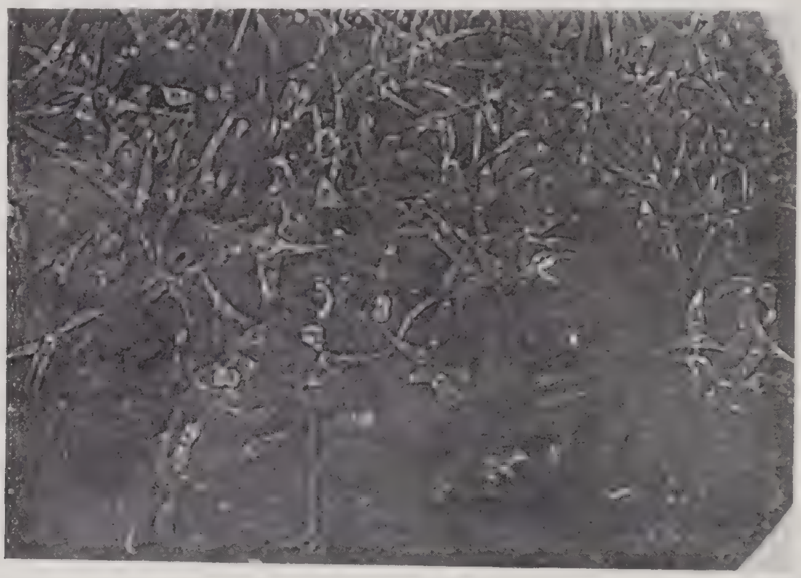


Fig. 2.3
Photograph of living cells in a culture of chick heart.

Occasionally, in parts of a culture or even throughout the whole outgrowth of a culture from chick heart, the migrating cells may be of a different form. They may appear in the form of a sheet of very large flattened cells connected together on all their surfaces of contact in much the same way as the cells of a pavement epithelium are arranged. These cells often show a modified region of the cytoplasm near the nucleus—called the centrosphere. Lewis (1923) has described all stages of transition between the two forms of cell, i.e. a mechanocyte may transform into a flat "epithelioid" cell; and, *vice versa*, epithelioid membranes have been observed to break up into a network of cells of the "mechanocyte" type. Furthermore, cells of the mechanocyte type though with somewhat different cell-processes, have been observed to contract rhythmically like normal cardiac muscles: this they may do either as isolated units or in groups (Lewis and Lewis, 1924; Rinaldini, 1958). Sometimes cross-striations are visible in the cytoplasm, but they do not appear to be necessary for normal rhythmic contraction (Fig. 2.4). These observations suggest that the cells which migrate out from the heart include not only true cardiac muscle cells, hence the beating cells, but also endothelial or mesothelial cells, for this would be the simplest explanation for the "pavement" behaviour. Moreover, when heart cultures in which the cells are growing as a network, are allowed to grow for a few days, particularly in a medium containing blood plasma into which the cells penetrate, and they are then treated by silver methods for the demonstration of reticulin or argyrophil fibres, it will be found that such fibres, which are normally regarded as the products of connective tissue cells (true fibroblasts and fibrocytes), are present in the interstices between the cells of the "mechanocyte"

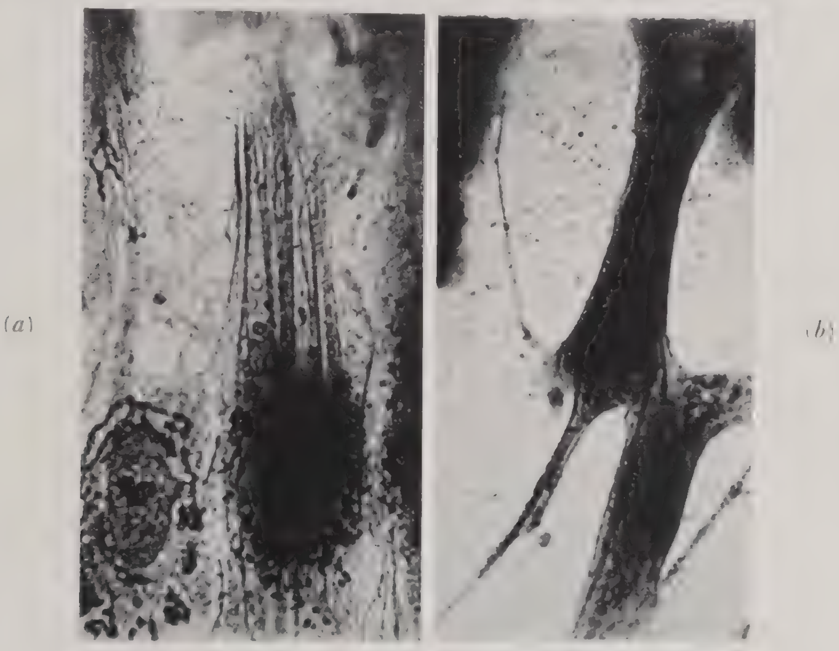


FIG. 2.4
(a) Heart muscle cell, showing striations in a seven-day-old culture of chick heart. (b) Two heart muscle cells in a two-day-old culture of chick heart. Each was pulsating with its own rhythm. Striations not apparent. (Lewis and Lewis, 1924.)

network. Under some conditions these fibres are transformed into typical collagen fibres, and elastic fibres also may be found to have developed (Bloom, 1930). True fibroblasts may thus be present in the outgrowth, as they are certainly present in the original tissue. Two explanations of the behaviour of the cells in the outgrowth seem therefore to be possible. Since, apart from macrophages, the outgrowth of cells may actually include cells of all the main classes present in the original tissue, heart muscle, connective tissue, endothelium and perhaps mesothelium, it is possible that the apparent uniformity arises because some or all of them can change their behaviour, e.g. cardiac muscle cells may actually become fibroblasts and so be capable of forming fibres: in other words, heart muscle, fibroblasts and endothelium may all grow *in vitro* as "mechanocytes". Alternatively the cells which emigrate and survive may belong to one category only, i.e. only the fibroblasts flourish under tissue culture conditions. This, however, is inconsistent with the observations on the contractile cells and of the epithelioid type of behaviour, for both these may persist in the outgrowth for many days (Fig. 2.5), and show no obvious signs of necrosis or of being overgrown by the "fibroblasts". It is therefore probable that the three original types of cells, with their many different physiological characteristics, all become morphologically extremely similar, if not actually indistinguishable under tissue-culture conditions. As already indicated, this morphological "dedifferentiation" of the cells of the heart raises far-reaching problems as to its significance and as

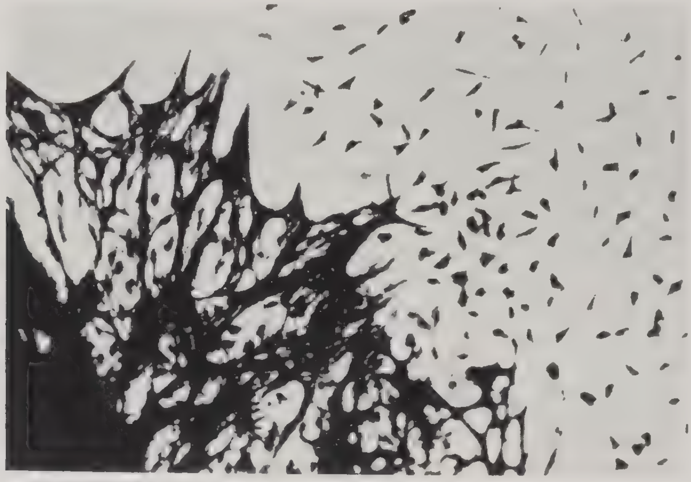


FIG. 2.5
Heart muscle (left) and other cells (right) in a five-day old culture of chick heart. (Lewis and Lewis, 1924.)

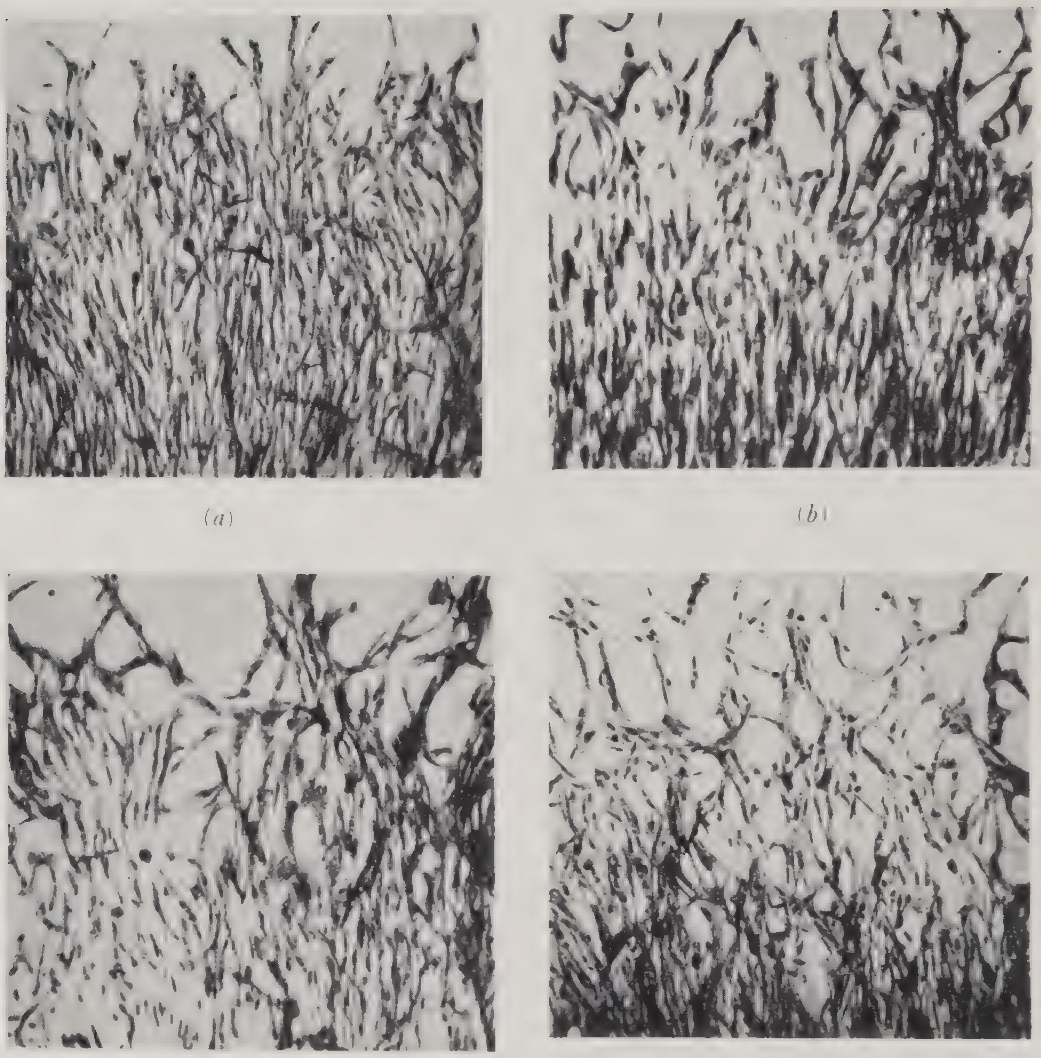
to the bearing which it may have on physiological dedifferentiation.

When fresh heart muscle from a developing chick is treated with a dilute solution of crude trypsin or of elastase in a calcium- and magnesium-free physiological salt solution (e.g. Tyrode's solution containing sodium citrate in place of $CaCl_2$ and $MgCl_2$), the living cells can be made to fall apart relatively undamaged (Rinaldini, 1958). Suspensions can be made from these cells in a plasma medium which is then allowed to clot so that the cells, completely isolated from each other or in only very small aggregates, are scattered and suspended throughout the coagulum. Under such conditions and with proper precautions against damage to the cells, most of the cells survive and, though they appear round and formless at first, they quickly spread out and several types of cell can be recognized. Eventually, however, they mostly assume essentially mechanocytic characteristics. Cells can frequently be observed to beat while still in complete isolation. In the early stages of such cultures, therefore, some undoubted heart-muscle cells are visibly separable from other cells which might originate from the connective tissue elements of the original tissue; the contractile cells stain rapidly with the intravital dye, neutral red, but after a few days such differences disappear and the whole culture appears to consist of mechanocytes; contractility may, however, still be displayed by many of the clusters of cells. It is not clear yet what happens to the cells between the stage of their being recognizable as heart muscle and the stage when the cells all appear to be mechanocytes. Some death and autolysis of cells may occur but it is unlikely that this explains events better than does a transformation of muscle cells to the morphology of mechanocytes, especially in view of the frequent persistence of contractility in the cell groups.

The Nature of Mechanocytes

Not only may such cells, i.e. mechanocytes, arise from fragments of chick heart, but, as already indicated, cells which habitually appear the same in tissue culture and which also behave in a very similar way, emigrate from fragments of many other tissues, e.g. from the walls of blood vessels, from loose connective tissue, from developing or adult bone, from perichondrium and periosteum, from skeletal muscle and the muscular coats of the intestine, and from many other sources. Since nearly all these sources are from tissues which are concerned with the structural and hydraulic systems of the body, the term "mechanocyte" seems to be appropriate (Fig. 2.6).

The view that such "mechanocytes" are derived from only one type of cell (e.g. the fibroblast, or fibrocyte, of the connective tissue present



(a) (b) (c) (d)

Fig. 2.6
Growth of mechanocytes derived from four different sources. (a) Muscle. (b) Chondroblasts. (c) Heart muscle. (d) Osteoblasts. (Fischer, 1930.)

in the original explant of tissue), while attractive in its simplicity, has already been shown to be improbable in the case of cultures from the heart. In cultures of skeletal or striated muscle, the muscle fibres can be observed to split up into individual myoblasts (de Rényi and Hogue, 1934) (Figs. 2.7, 2.8), eventually to become, morphologically, mechanocytes. This splitting up of the muscle fibres is often preceded or accompanied by the presence of peculiar multinucleate muscle-buds probably formed by the migration of the nuclei into the ends of the fibres

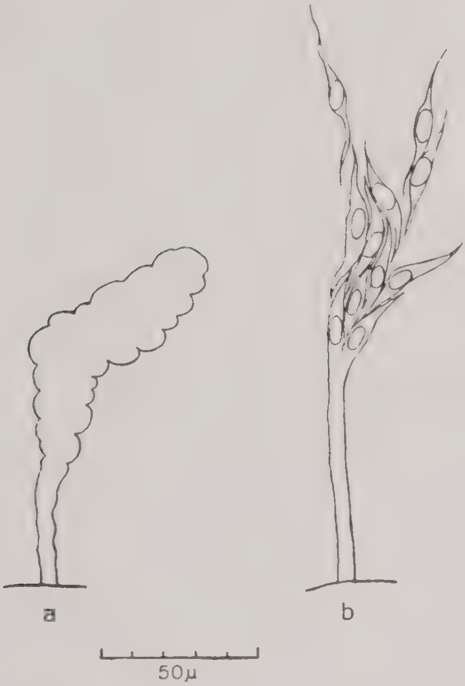


Fig. 2.7
(*a*) A 'muscle-bud' in the outgrowth of a tissue culture of limb muscle of a chick embryo. (*b*) The same 'muscle-bud' splitting into myoblasts twenty-four hours later. (de Rényi and Hogue, 1934.)

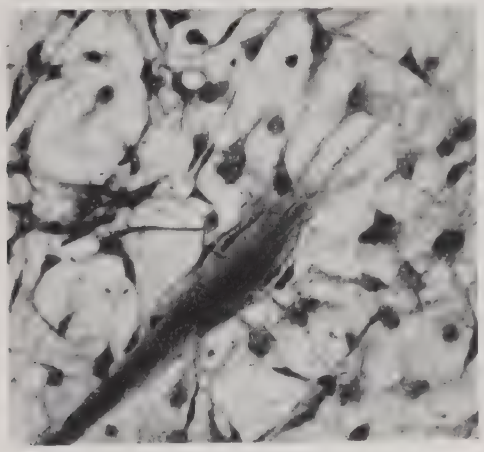


Fig. 2.8
A 'muscle-bud' splitting into myoblasts. (Lewis and Lewis, 1917.)

(Fig. 2.9). It should, however, be mentioned that certain other types of cell (see p. 96) may also emerge from cultures of muscle and it is possible that muscle is not the uniform tissue which it appears to be at first sight. Indeed, the formation of multinucleate cells is a characteristic of another group of cells (see p. 92), and it is not every muscle fibre in a culture which shows this curious form of behaviour.

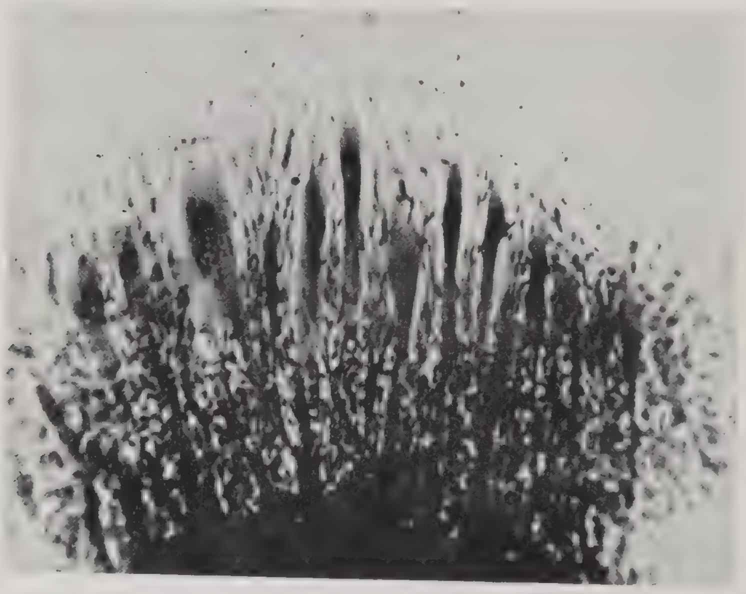


Fig. 2.9
Multinucleate cell masses developing from muscle fibres in a two-day old culture. (Lewis and Lewis, 1917.)

In addition to the evidence of this sort against the uniformity of origin of the mechanocyte population in a tissue culture, there are also further arguments which can be raised against it. There is good evidence, for example, that mechanocytes in tissue cultures, though morphologically similar, are not all physiologically identical. Mechanocytes of different origin do not all grow at the same rate, but in any given medium each has its own characteristic rate of growth and frequency of cell division (Parker, 1933b; Willmer and Jacoby, 1936). Moreover, the study of the ultimate fate of such cells, when their cultivation *in vitro* is successfully prolonged for several weeks or months, especially in conditions which favour their subsequent differentiation and functional activity, emphasizes their inherent differences. To encourage differentiation rather than growth, tissues may be planted in a serum medium on the flat bottom of special flasks (Carrel flasks) or they may be embedded in a plasma clot on the bottom of such a flask. At frequent intervals the cultures are washed with fresh serum.

or the surface of the plasma clot is irrigated with fresh plasma to which sufficient heparin has been added to prevent its coagulation, so that more food substances are added and metabolites are to some extent removed without increasing the depth of the clot (Fischer and Parker, 1929). Alternatively, they may be placed on the surface of a solid nutrient medium or supported by a raft of cigarette paper or rayon (cellulose acetate) on a fluid medium in an embryological watch-glass (Fell and Robison, 1929; Shaffer, 1956). Under such conditions the cells do not continue to emigrate indefinitely into the medium. After a brief period, there is very little more, if any, out-growth, and the cells in the central explant begin to differentiate. The new tissues which are then formed are recognizably similar in character to those from which the cultures were derived; they are quite stable and remain alive for long periods. For example, cultures of muscle have remained alive for about a year and could then still be activated to grow as free mechanocytes by transferring them to hanging-drop cultures in a growth-promoting medium. Such cultures when sectioned and

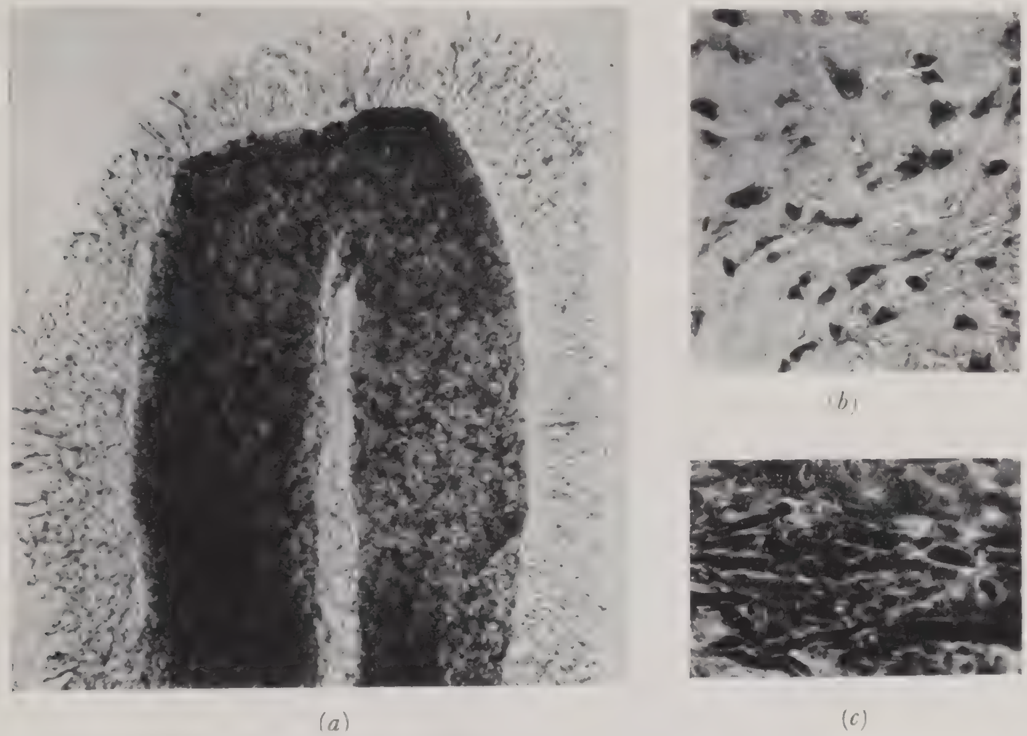

(a) (b) (c)

FIG. 2.10

(a) Outgrowth of mechanocytes stimulated by the addition of embryo juice to a colony of muscle tissue kept in 'latent life' for a year. (b) and (c) Sections through the central explant of a muscle culture kept in 'latent life' for a year, (b) showing collagen fibres and (c) showing a few muscle fibres. (Parker, 1936.)

examined histologically, show the development of large amounts of connective tissue fibre and of occasional muscle fibres (Parker, 1936) (Fig. 2.10).

Admittedly, the relative increase in connective tissue and the decrease in amount of muscle is a little disturbing, but possibly, the conditions under which muscle develops *in vitro* are rather more complex than those required for the formation of collagen fibres, for these seem to be produced rather easily, first as argyrophil fibres and then as true collagen. Moreover, muscle is a tissue which does not readily regenerate *in vivo* and also tends to atrophy with disuse, so it would be surprising if it did better *in vitro*.

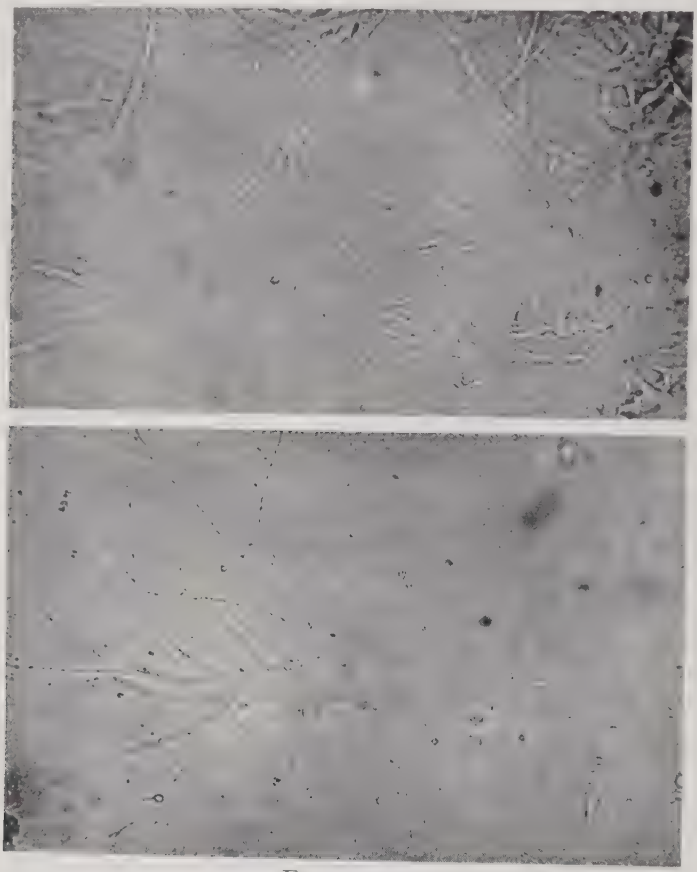


FIG. 2.11
'Fibroblasts' in the outgrowth of colonies of muscle tissue kept in 'latent life' for a year. (Parker, 1933a.)

The cells which remain in the periphery of these relatively dormant cultures often begin to differentiate also and to assume forms quite unlike the usual mechanocytes seen in cultures treated with the growth-promoting embryo juice (Parker, 1933a) (Fig. 2.11); the precise identification of such cells is, however, uncertain, but their behaviour makes it clear that "mechanocytes" are certainly capable of differentiating in a variety of different ways or at least into a variety of different forms.

Another example in which the true nature of the cells in culture can be determined by their capacity to re-differentiate is very illuminating. When cultures of mechanocytes are obtained from fragments of bone taken from the shaft of a femur previously deprived of all adherent cells (periosteal, endosteal and those from the bone marrow), presumably the cells emerging in such cultures are likely to have come only from bone cells (osteoblasts, osteocytes or osteoclasts), or from the cells associated with the developing Haversian systems and blood vessels (Fig. 2.12). The cells in the outgrowth of these cultures mostly behave like the cells from the heart, i.e. like mechanocytes: they migrate out copiously, with the same sort of movement, and form a network of spindle-shaped or fan-shaped cells. After a time, the original explant of bone can be removed, and the space which it occupied can be filled with fresh plasma coagulum. The surrounding cells immediately begin to grow back into this vacant space, especially if the medium as a whole is growth-promoting and composed of a mixture of plasma and of extract of embryo tissues in physiological salt solution (i.e. embryo extract). When the space has become entirely filled with cells the concentration of the growth-promoting substances in the medium is reduced by decreasing the concentration of embryo extract. Under these conditions the cells in the centre, which are, of course, derived

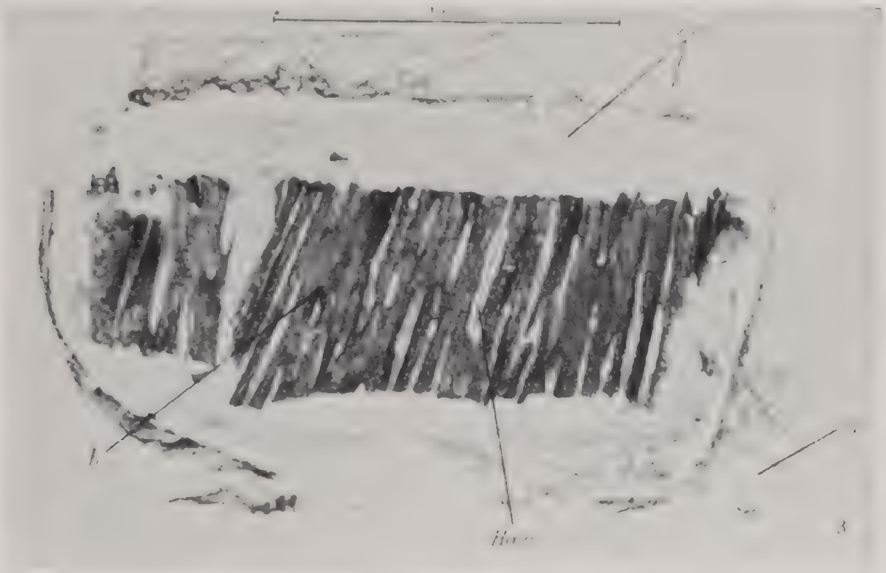


Fig. 2.12

Culture of endosteal bone from the tibia of a chick, showing the original explant (b) and the zone of outgrowing cells (z.o.). Ha.c., Haversian canal. (Fell, 1933.)

from those which originally migrated away from the bone, now begin to differentiate. In most cases, the cytoplasm of the cells becomes hazy in appearance, a ground substance or matrix is laid down between the cells and this matrix then calcifies; histological sections of the differentiated tissue show that osteoid tissue or even true bone has been formed (Fell, 1932) (Fig. 2.13). The cells therefore which had

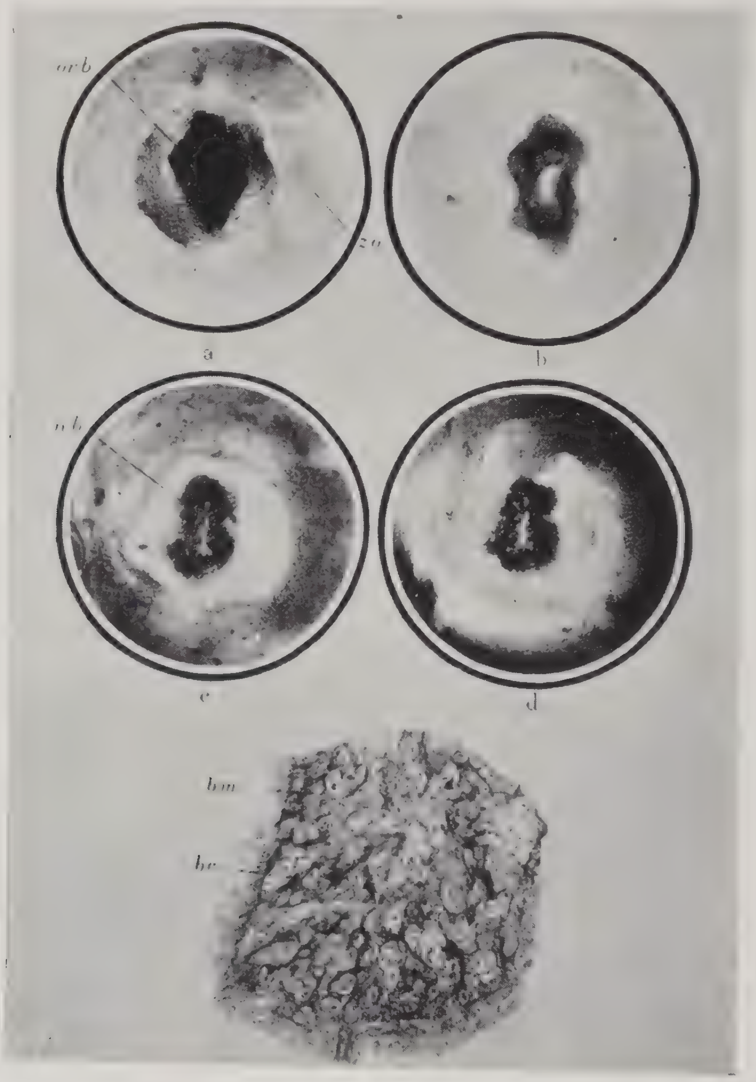


Fig. 2.13

Development of bone from mechanocytes. (*a*) Original culture showing outgrowth of mechanocytes. (*b*) Original explant removed, with retraction of the zone of outgrowth. (*c*) and (*d*) New bone developing in the centre of the colony. (*e*) Section of the newly formed 'bone'.

bc—bone cell. bm—bone matrix. nb—new bone. or.b—original bone. zo—zone of outgrowth.

(Fell, 1932.

emigrated and were behaving as generalized mechanocytes in the original outgrowth from the bone fragment must have contained among their numbers at least some potential bone cells (osteoblasts and osteocytes); moreover, the frequency with which the differentiation of bone occurs in such cultures suggests that bone cells are normally numerous in the original out-growth and must therefore constitute a large proportion of the mechanocytes present.

Although, as we have seen, the mechanocytes in cultures from heart or muscle tissue, and, in fact, in cultures from many tissues other than bone, may differentiate under conditions of decreased growth-stimulation and form fibrous tissue, and although precipitates of calcium salts may occur in some of these cultures, e.g. as in cultures of heart tissue under some conditions, yet true bone with its characteristic fibrous pattern and crystalline structure only differentiates from mechanocytes derived from tissues originally possessing osteogenic potentialities. On these grounds therefore, the osteogenic mechanocytes must be considered as a special breed, and physiologically different from the muscle mechanocytes.

Since bone cells, heart cells and cells from the other sources indicated (see p. 25) all seem to change their morphological characteristics when they emigrate in tissue culture, and since, in the case of the heart muscle treated with trypsin, various other morphological forms appear in the early stages, it may be asked whether there is any real significance to be attached to the mechanocyte form, other than that it is the characteristic form which cells take up under certain conditions of tissue culture, particularly those which favour the migration of cells and their growth by cell-division. In later pages it will become clear that this form almost certainly has a biological significance and indeed a very fundamental one, but, at this stage, it must suffice to say that many tissues such as those already listed, habitually and regularly produce mechanocytes *in vitro*. By way of contrast, however, it must be emphasized that cells from certain other sources of tissue, just as habitually and just as constantly, behave in other equally characteristic ways and do not normally, if ever, assume the mechanocyte form. They may perhaps be able to do so under certain special conditions, but the evidence for such transformations is often equivocal and in one or two cases will be the subject of later discussions.

Although tissue culture teaches, above all things, that too much accent must not be placed on mere outward morphology, the mechanocyte form is nevertheless a definite and recognizable form of cell appearance and characterizes the behaviour of cells derived *in vitro* from certain well-defined sources. Furthermore, it will be shown that this cell-form is connected with a particular pattern of behaviour, and that the mechanocyte has a definite series of potentialities for further differentiation, when suitable conditions are provided to evoke them.

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CHAPTER 3

THE GROWTH OF MECHANOCYTES

THE general morphology, the orientated gliding movement, the capacity to form argyrophil and collagen fibres (for which reason the original term "fibroblast" is not altogether inappropriate), and the property of linking up with its neighbours to form a cellular network have already been stressed as diagnostic characters of the mechanocyte. So too are the special conditions which favour active growth by cell division among these cells.

When embryo tissues from a wide variety of sources—particularly those already mentioned, e.g. heart, periosteum, bone, connective tissue etc.—are cultured in media containing plasma and embryo extract and the medium is frequently renewed, exuberant colonies of mechanocytes are obtained. The embryo-tissue juice (or extract) is an essential factor in this growth: it both stimulates the movement of the cells, so that they migrate more quickly into the medium, and at the same time it fosters and maintains cell division. Cells divide more frequently and more quickly in its presence. In fact, with suitable renewal of such media, cells may be kept alive and growing for an indefinite time *in vitro*, and in the outgrowth of such cultures it is not unusual to record mitotic rates of more than 5%: that is to say, that more than 5% of the cells may be seen in mitosis at any one time. Since mitosis generally lasts for a little under an hour; this means that approximately 5% of the cells divide per hour so that the population, as a whole, may double itself within a day or so. These growth-rates can be conveniently recorded by time-lapse cine-microphotography directly on to sensitive paper. Only one particular zone of the explant can, unfortunately, be examined in this way at any one time, but repeatable results can be readily obtained. The frequency of the exposures (e.g. 6 minute intervals) is arranged so that there is little chance of missing any of the cell divisions occurring in a population of several hundred cells. The method, of course, gives no information concerning the cells in the centre of the explant, and can only be applied when the cells are spreading in a single layer (Willmer, 1933a), and are thus under distinctly unnatural conditions.

If the embryo juice is omitted from the medium, then this exuberant growth does not continue after the first few hours and photographic records show that mitosis practically ceases in the outgrowth after something of the order of 48 hours, and that cell movement only continues thereafter at a very slow rate (Willmer, 1933b) (Fig. 3.1).

Recent work on clones and pure strains of cells, particularly when these are grown on more or less synthetic media has indicated that after prolonged culture *in vitro* mechanocytes, and indeed other cells also, may acquire new properties (Sanford, Likely and Earle, 1954; Parker, 1957; Puck and Fisher, 1956). For example, cells which were originally mechanocytes, and as such would need embryo extract, have now been kept actively multiplying for many months on a synthetic medium without any embryo juice (Healy, Fisher and Parker, 1954b; White, 1949). It is probable, however, that in such cases the cell colonies actually have acquired new properties in response to changed conditions in much the same way as bacteria "adapt" to new media, etc. A beautiful example of such a spontaneous change of character is illustrated in Fig. 3.2. The culture originally consisted of a clone (i.e. a culture started from a single cell) derived from the epithelial cells of the kidney. After a time, certain nests of cells spontaneously appeared in which the cell morphology was entirely different and in which the cells continued to breed true in their new form (Parker, 1957).

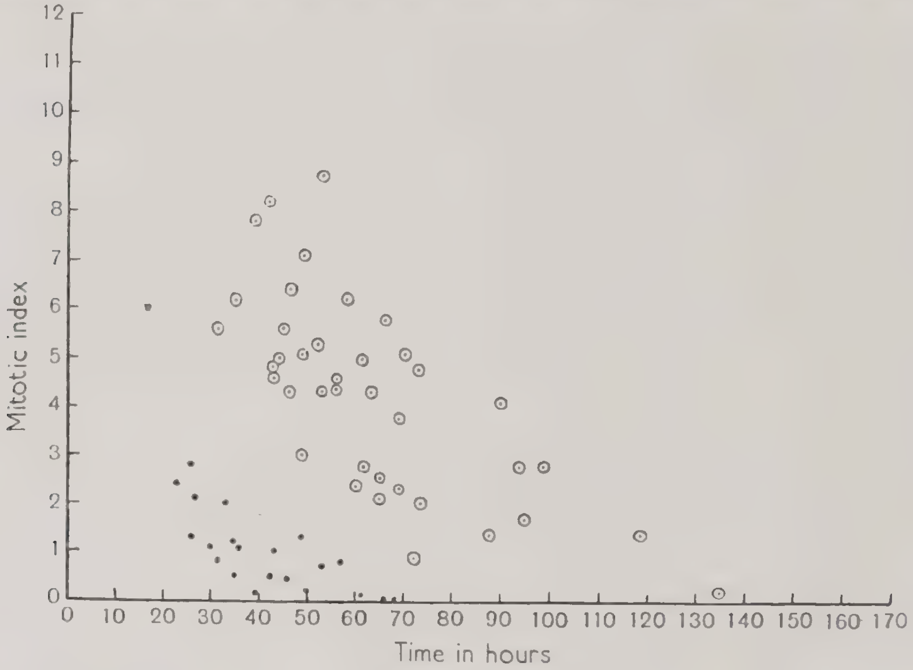

FIG. 3.1

Mitotic index plotted against age of the culture in a medium containing embryo extract (o) as compared with that in a medium of plasma only (·). (Willmer, 1933b.)

Incidentally, this new form is quite typical of that adopted by many types of cell after prolonged culture in "pure strain".

The type of behaviour of cells now under discussion, however, concerns cells recently derived from the animal and with as little "adaptation" to tissue-culture conditions as possible. Even though migration and mitosis almost cease in the plasma medium after a few days, the cells are still perfectly healthy; indeed, if washed periodically with serum, they may remain alive and functional for years. From the point of view of growth, however, they are quiescent, and are now in a condition very suitable for the investigation of the effects of potential growth-promoting media, which may be added at this time, i.e. say 60 hours after explantation into a medium consisting of plasma only. These quiescent mechanocytes respond particularly favourably to treatment with embryo juice; with it they begin to grow again: without it they normally do not grow—though they may, as indicated above, survive for very long periods and though they may, under special

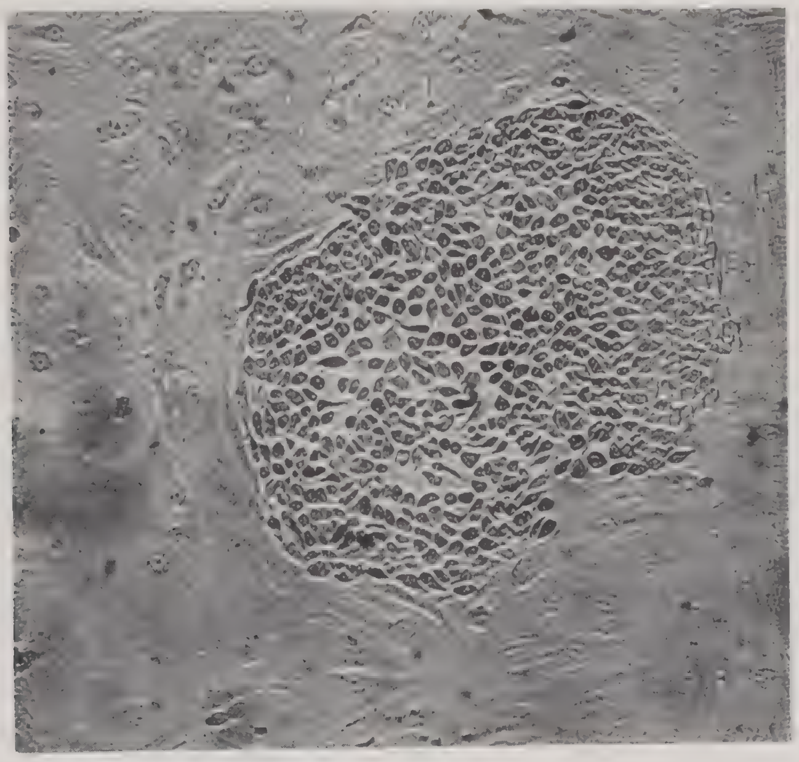

Fig. 3.2

A nest of altered cells in a clone derived by single-cell isolation from a culture of the kidney epithelium of a monkey. The unaltered epithelial cells can be seen surrounding the nest of altered cells. (Parker, 1957.)

conditions, "adapt" and then grow spontaneously. Within two hours of the addition of the extract, cell movement is measurably accelerated and a velocity of 12μ per hour may rapidly climb to 40μ or even 50μ per hour (Willmer and Jacoby, 1936) (Fig. 3.3). There is a lag period of about 10 hours before cell division is resumed. This lag period always occurs and is probably connected with the building up of the nuclear deoxyribosenucleoprotein (see p. 40).

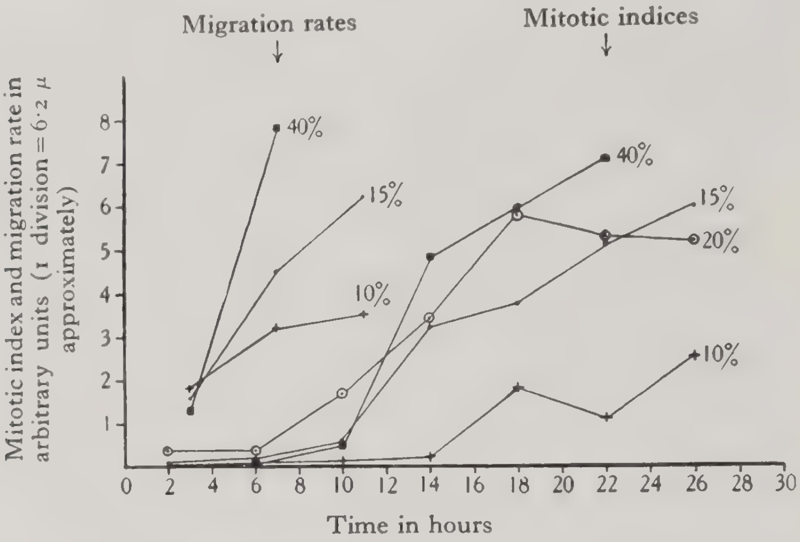

FIG. 3.3

Diagram showing the almost immediate increase in migration rate, and the delayed increase in the mitotic rate when different concentrations of embryo juice are added to cultures of 'osteoblasts' reduced to quiescence by previous culture in plasma only. (Willmer and Jacoby, 1936.)

It is immaterial from which of the various sources mentioned above the tissue is derived; any growth of mechanocytes of which the tissue is capable always seems to be activated to a greater or less extent by the presence of embryo extract, and mixed colonies of cells from complex tissues often end up, after continued cultivation and subcultivation in its presence, as apparently pure colonies of mechanocytes. On the other hand, in spite of this general sensitivity to embryo extract there is evidence that mechanocytes from different sources respond quantitatively differently (Parker, 1933). Embryo juice can be conveniently prepared by washing 7-10 day chick embryos free from blood, yolk, etc., crushing them to a pulp, and then centrifuging away the tissue debris. The opalescent supernatant fluid may then be regarded as 100% embryo juice which can be diluted with Tyrode's solution (or other suitable medium) to any desired concentration. The age of the chick from which the juice is made is of some importance and should be standardized as far as possible in any given series of experiments. It has then been found that, when such dilutions are applied to quiescent cultures of races of mechanocytes from different sources, the optimal concentrations are quite different. For mechanocytes from the frontal bone of the chick, 40% juice produces decidedly greater rates of growth than 15% juice and full growth-stimulation does not occur till the concentration is raised to this upper limit. On the other hand, in mechanocytes derived from the heart of the chick the maximum mitotic rates are produced by 15% juice and these are generally rather lower than those occurring in the tissue cultures derived from periosteum under the same conditions (Willmer and Jacoby, 1936). Moreover, when the concentration is raised above 15% there is an increasing tendency for some races of mechanocytes, e.g. those from the periosteum, to digest or liquefy the supporting plasma coagulum.

These experiments again emphasize certain common properties shared by mechanocytes, but at the same time they show that, within the mechanocyte "family", there may be many races or genera.

Some interesting sidelights are thrown on the behaviour of mechanocytes by experiments in which the embryo juice only remains in contact with the tissue for a limited time (e.g. 1 hour) and is then removed and replaced by Tyrode's solution (Jacoby, Trowell and Willmer, 1937) Under these conditions, mitoses occur after the usual latent period (10-12 hrs.) and their number depends on the concentration of the embryo juice. They now, however, cease to occur after a further period of about 12 hours, instead of continuing for several days as they would if the juice were left in contact with the cells (Fig. 3.4). Repetition of the treatment with the juice can again produce mitoses after 10 hours, but this only occurs when the second dose is given after the first dose has already become effective, i.e. if given more than 10 hours after the first dose. When it is applied earlier than that, i.e. during the "latent period" of the first dose, then the second dose is ineffective. The interpretation would seem to be that the active agents in the extract penetrate the cell quickly, i.e. within the hour, and alter its metabolism in such a way that the movement of the cell is visibly more active within the first two hours, and metabolic processes are inaugurated, which finally lead up to cell division after a period of ten or more hours. Those cells which have been activated are not susceptible to further treatment with embryo juice until the first division has occurred, but then they may be re-charged, so to speak, and produce a second outcrop of mitoses after another lag period of about 10 hours. Such evidence as there is encourages the belief that the cells of the second crop, as implied above, are actually the daughter cells from the first crop. Since high concentrations of embryo juice produce numerous mitoses in these experiments, while the numbers of divisions are much less in low concentrations, it is probable that there are in a colony of mechanocytes, cells whose sensitivities to the stimulating factors of embryo juice differ. There are some which are, as it were, nearly ready to divide and only need say 5% juice in the medium to send them into division, while others are much further from the division state and require 15% or even 40% juice before they are raised to the pitch at which they can respond. From the point of view of growth, therefore, an apparently uniform population of cells may, in fact, be anything but uniform. Moreover, the cells which respond by division to weak concentrations of juice are scattered in an apparently random manner throughout the outgrowth, so that it is not simply a question of the availability of limited quantities of some activating substance.

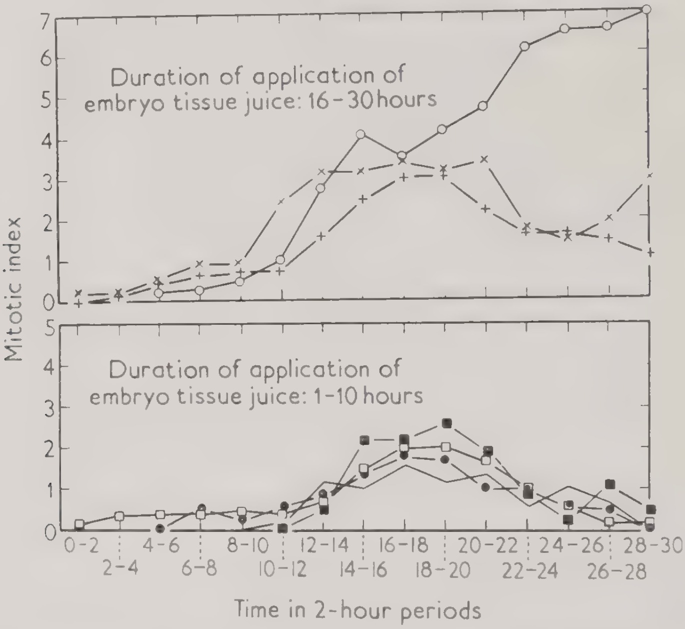

FIG. 3.4

Curves showing the effect on the mitotic index of applying embryo juice to cultures of periosteal fibroblasts for different lengths of time, from 1 to 30 hours.

⊙—⊙ 30 hrs. ×—× 20 hrs. +—+ 16 hrs. ■—■ 10 hrs.
.—. 6 hrs. □—□ 3 hrs. ——— 1 hr.

(Jacoby, Trowell and Willmer, 1937.)

There is evidence that ribosenucleoproteins (RNP) are concerned in this activation, for, from measurements of the ribosenucleoprotein phosphorus or of the ribosenucleic acids (RNA), they appear to be taken up or formed within the cell rather quickly after the addition of embryo juice, and are then probably instrumental in increasing the deoxyribosenucleoproteins (DNP) of the nucleus preparatory to division (Willmer, 1942; Davidson, Leslie and Waymouth, 1949). Studies of the deoxyribosenucleic acid content (DNA) of tissue-culture cells indicate that the amount present at division is constant, but that between divisions it is significantly less, presumably because at division the amount is halved between the daughter cells and these then build it up slowly to the "tetraploid" amount (Walker and Yates, 1952). The relationship between the DNA content of the nucleus and the type of cell is obviously very important in connexion with the nature of cell differentiation and the ability of races of cells to breed true (Healy, Fisher and Parker, 1954a). Fibroblasts under the influence of embryo juice and plasma normally breed true and do not give rise to other types of cell at all readily. It is not yet known whether this capacity to breed true is a nuclear or a cytoplasmic phenomenon. If it is nuclear, how is the DNA content of the different races affected, or is the difference between races dependent on some difference in the protein part of the chromosomes, or in the RNA of the cells? Just as slightly different properties are imparted to different haemoglobins by variations in the protein to which the haem is attached, or to visual pigments by variations in the protein to which the retinene is attached, it is tempting to wonder if the properties of genes may be modified by the particular protein to which the DNA is attached. It is unlikely to be anything so drastic as a major change in chromosome number or a fundamental alteration in the DNA because, although the races of cells in an organism may differ from each other and breed true as such in culture, they still remain species specific. It is true that the chromosomes in somatic cells of the chick are peculiar in that they show a tendency to fragment, but it may be of interest to observe that the number of fragments visible in the mitoses of chick mechanocytes in tissue culture is in general greater than the number of fragments in the macrophages of the same species under similar conditions, thus perhaps indicating some difference in the constitution of the chromosomes in the different somatic cells.

In the presence of embryo juice, even in very small amounts, the growth of the mechanocytes is greatly accelerated by the presence of proteoses, especially when these are derived from fibrin by partial digestion with pepsin (Carrel and Baker, 1926; Baker and Carrel, 1928). The fact that the digest of fibrin is better than that of other proteins is perhaps a point of physiological importance. In the first place it indicates that certain groupings of amino acids are probably more suitable than others; but it also suggests that the fibrin deposited in a wound in the body may not be merely an inert scaffolding in relation to the subsequent repair process in which mechanocytes, as potential tissue cells, play a large part. Furthermore, the fibrinogen circulating in the blood and lymph perhaps has potentialities as a normal food substance for the mechanocytes and their derivatives in the body and is not only concerned with the clotting of the blood or lymph. This appropriateness of fibrinogen as a nutrient for fibroblasts is worthy of further study and should be borne in mind in relation to theories of the origin of "fibroblasts" (see pp. 95 and 238).

In the absence of embryo juice the proteoses are apparently much less effective (Willmer and Kendal, 1932). It is as though the embryo juice increased the protein metabolism, and the presence of proteoses in the medium provided a raw material or substrate which is far more effective than either whole proteins or most amino acids. However, the embryo extract also accelerates the uptake of glucose from the medium (Willmer, 1942), so the mode of its action is anything but clear; nor is it yet possible to make any concentrate from it which is more active than the extract as a whole. Probably there are several factors concerned, some at least of which are thermolabile and non-dialysable (perhaps lipoproteins or ribosenucleoproteins); others are thermostable and dialysable and probably include certain amino acids (Baker and Carrel, 1926a, 1926b; Fischer, 1941; Harris and Kutsky, 1954; Hueper *et al.* 1933; Jacoby, 1937; Lasnitski, 1937).

The whole question of growth-stimulation by embryo extract, proteoses, etc., has been treated at some length here, because it is particularly relevant to the behaviour of mechanocytes, which, unlike certain other cells (see p. 86), will normally not continue to divide, after the first few hours, in a plasma medium devoid of embryo extract; they can, however, be brought once again into a state of division, even after they may have been dormant for very long periods *in vitro* in a medium of plasma or serum, if they are treated with embryo juice. Even in the tissues from an adult organism, where the fibroblasts must have been relatively dormant for years, they again become active when the tissue is treated for some time with embryo juice in tissue culture conditions. The effects can be accelerated by previous treatment of the tissue with solutions of trypsin (Simms and Stillman, 1937). The discovery of the effects of embryo juice was made by Carrel in 1913 and there is still nothing more effective in causing cell divisions among mechanocytes, though saline extracts of some adult tissues, e.g. brain, thymus (Trowell and Willmer, 1939), cardiac muscle (Doljanski and Hoffman, 1939), and cartilage (Davidson and Waymouth, 1943), show some similar but weaker activity. It is very difficult to correlate the capacity of a tissue to provide saline extracts of high growth-promoting quality with any other metabolic features possessed by that tissue (Pomerat and Willmer, 1939). High ribosenucleoprotein content and high glycolytic activity, especially under aerobic conditions, seem to be features common to many of the active tissues, but whether these features are more than incidental is doubtful. The high mucoprotein content of some of the active extracts is possibly significant.

It should be remembered that, in order to test the growth-promoting activity of tissue extracts, these are usually left in contact with the cells for a considerable time, and any toxic properties which they may possess are thus also given every opportunity to show themselves. Moreover, saline extracts of some tissues must inevitably contain substances which are inhibitory to cell activity, so that the total effect of each tissue extract must be the resultant of the growth-stimulating and the growth-inhibiting actions of the substances extracted. For example, in extracts of both liver and kidney either the inhibitors preponderate or there are no growth-promoting agents present, for these extracts are usually ineffective in stimulating the growth of fibroblasts. Only a fine line of distinction can be drawn between inhibitors and toxic agents. Reversibility of the effect is perhaps the simplest criterion. There are, however, several substances which more or less reversibly suppress the activity of mechanocytes, and some of these (though not all, of course) act specifically on mechanocytes, leaving other tissues active and apparently unaffected. For example, when certain aldehydes (0.002M) are added to the culture medium of mechanocyte colonies, the peripheral cells rather quickly withdraw their processes and round up. They may remain apparently inert in this condition for days, but as soon as the aldehyde is removed they spread out again and go on growing as before without any permanent ill-effects. Glyceraldehyde and propylaldehyde are particularly effective (Pomerat and Willmer, 1939). The lowest aliphatic aldehydes, formaldehyde and acetaldehyde, are, as might be expected, toxic. Many of the sulphonamide drugs, in relatively high concentrations, act much in the same way, and, again as might be expected, the more soluble they are the more effective they are (Jacoby, Medawar and Willmer, 1941). A particularly interesting inhibitory substance in relation to the present discussion is found in malt extracts (Heaton, 1926; Medawar, 1937), and has been identified as a hexenolactone (Medawar, Robinson and Robinson, 1943). This substance may entirely suppress the activity of mechanocytes while leaving cells of some other types (e.g. epithelium) in similar cultures apparently untouched (Fig. 3.5). When applied to the whole animal the effects are, as might be expected, more complex

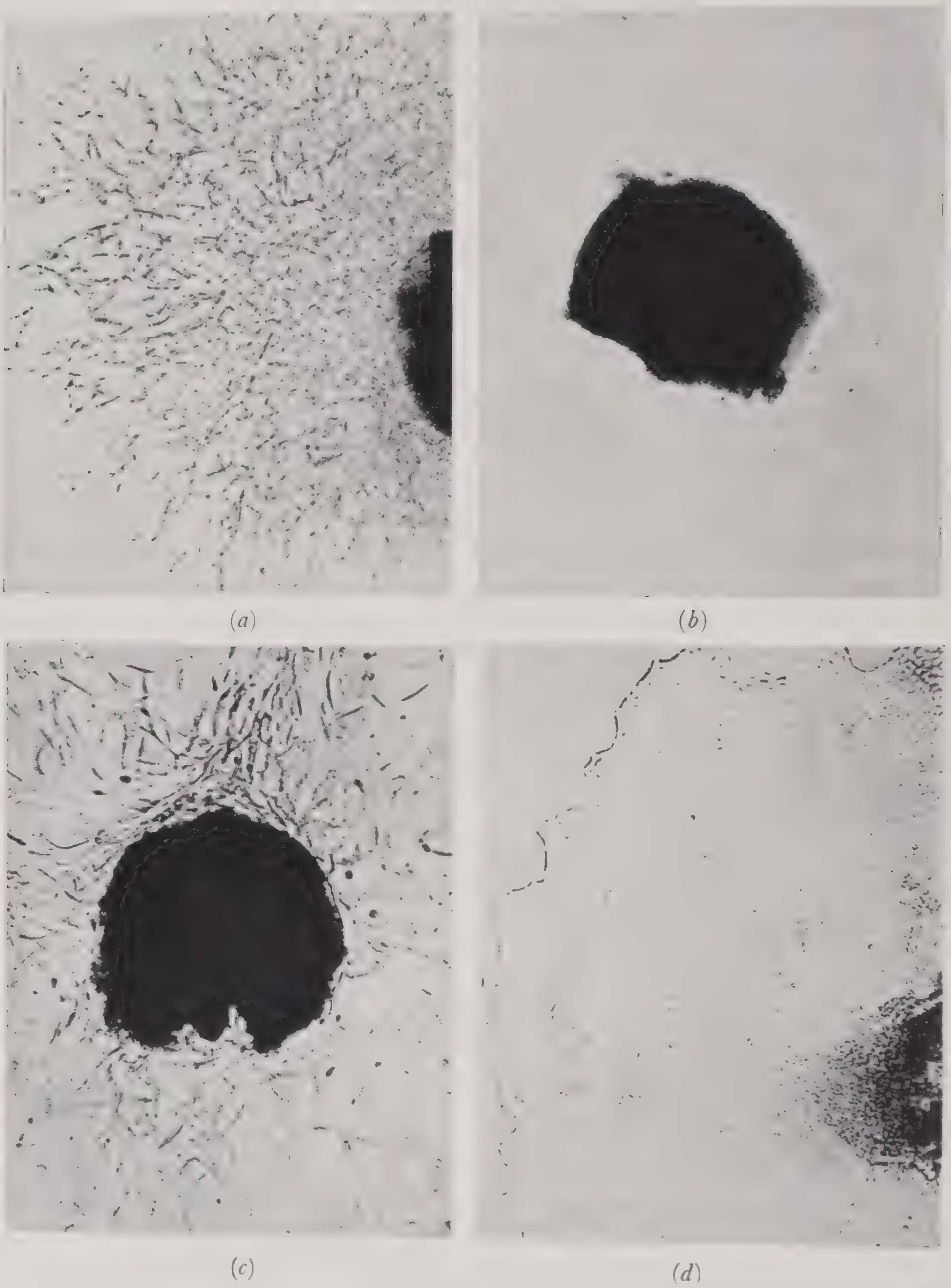

Fig. 3.5

Figures showing the inhibition of mechanocytes by a substance present in malt and maize extracts. (*a*) Normal growth of mechanocytes. (*b*) Comparable culture with inhibitor. (*c*) Growth of a culture like that in (*b*) after removal of inhibitor. (*d*) Growth of epithelium in medium containing inhibitor. (Medawar, 1937.)

(Briggs, 1946; Hauschka, 1946), and even in tissue cultures the distinction between mechanocytes and epithelial cells in their sensitivity to hexenolactone has been challenged (Royle, 1945). Other factors like cysteine, which reduces the toxicity, and glutamic acid, which increases it (Hauschka, 1946), are probably involved; serum has a protective action on the cells.

'PURE' STRAINS OF MECHANOCYTES

If there are different races of mechanocytes and since most cultures must contain mixtures of these different kinds of cells, or of cells in different physiological states, it is, at first sight, surprising that the making of pure cultures of the different breeds of mechanocytes from single cells has been so long delayed. The main reason which delayed this approach emphasizes a peculiar property of mechanocytes, namely that they survive and grow only with great difficulty in isolation. The typical network-like growth of these cells has already been emphasized and it is comparatively rarely that a cell leaves this network and survives to divide as an independent unit. Successful pure colonies from single cells of mechanocyte-type have been made by Earle and his school (Sanford, Earle and Likely, 1948; Likely, Sanford and Earle, 1952) by explanting single cells in media which have already supported colonies of cells, and also by Puck *et al.* (1956, 1957) who sometimes used "feeder" cells, i.e. cells whose growth had been stopped by irradiation, to initiate the growth of normal cells isolated on a layer of such cells. In the ordinary media, isolated mechanocytes, unlike one or two other types of cells, do not readily survive unless the cells are enclosed within a very limited quantity of medium as, for example, in a small capillary tube, or are given some other special treatment. It appears that the plasma-embryo juice medium, as normally used, is not itself entirely satisfactory for the rather fastidious mechanocyte, when it is completely isolated, but can be made so by the combined action of numerous cells, even when these are all of the same type, i.e. mechanocytes. The situation is somewhat parallel to the common failure of single fertilized echinoderm eggs to form blastulae when isolated on coverslips in hanging drops of sea-water, but their complete success when placed in hanging drops containing more than about 10 eggs. In this case, one of the factors is almost certainly the pH of the sea-water, which tends to become too high owing to solution of alkali from the glass; the CO_2 production by numerous cells can presumably counteract this, while that from a single egg may be insufficient. This is obviously a very simple consequence of the technique employed and other more complex factors are certainly involved in the tissue culture problem, though what they are is not yet known. Fresh mechanocytes which are growing on a coverslip in a completely fluid medium of embryo juice are very easily damaged by washing them even with fresh embryo juice, and one of the actions of a plasma coagulum in encouraging mechanocyte growth may well be the provision of a more constant local environment for the cells. On the other hand, the pure clones of cells originally derived from mechanocytes which are now being successfully cultured, can be kept in large volumes of agitated fluid media and the cells multiply freely under these conditions. (Earle, Schilling, Bryant and Evans, 1954). As discussed earlier, however, such cells should probably not any longer be considered as typical mechanocytes, but as "adapted tissue-culture cells". There is some evidence that myoinositol and perhaps other constituents of serum have a stabilizing action on these suspended cells (Eagle, Oyama, Levy and Freeman, 1957).

Synthetic Media

Not only has it been found difficult to establish cultures from single mechanocytes from normal tissues, but this family of cells is equally difficult to provide with a purely synthetic medium. Such media have been devised, and many of them are very complex. One of them initially contains 58 constituents in addition to water, though how much these interact and how many remain distinct in the final medium is another question. Nevertheless, they still do not support growth indefinitely. Probably not all these ingredients are essential but investigators in this field (Eagle, 1954; Eagle, Oyama and Levy, 1957; Healy, Fisher and Parker, 1954b; Waymouth, 1955; White, 1949) have considered it advantageous to incorporate in the basic medium all those substances which might be important to the cells, and then to discard them one by one in order to find out which are essential, rather than to build up the medium from the simplest necessities. The reason for this is that so often a combination of substances is more efficacious than any one of them alone and it is easier to spot these combinations subtractively than additively. While "organ cultures", i.e. those in which a complex tissue is encouraged to differentiate or function under tissue-culture conditions, can be successfully provided with synthetic media and often develop well on them for many weeks, the indefinite survival of pure strains of normal mechanocytes in the complete absence of traces of serum or plasma is more doubtful. So far the survival time of cultures of normal cells on purely synthetic media does not seem to be more than a few months, while in serum and embryo juice there appears to be no limit. The synthetic media have proved more successful with malignant cells or with cells which have become in some way adapted to tissue culture conditions. In any case, it is necessary to distinguish between survival, with or without differentiation, and growth.

These purely synthetic media naturally contain a large number of amino acids, either in more or less empirical amounts or in amounts based on the amino acids essential for blood formation or on the composition of digests of fibrin, etc. Mixtures of the L-amino acids are, on the whole, more effective than those of the racemic or D-forms. The effects of a few amino acids have been tried alone or in simple combinations and something positive is now beginning to be known about the requirements of mechanocytes in this direction. For a strain of cells originally derived from a colony of mouse fibroblasts (strain L) thirteen amino acids have been found to be essential (Eagle, 1954). However, the "normality" of these cells is suspect, owing to their tendency to become malignant, and further work is necessary on cells still in the normal mechanocyte state and less "adapted" to tissue culture conditions. Nevertheless it may be found to be very significant in relation to a later discussion (see p. 187) on the nature of mechanocytes that among the amino acids which are not essential for the growth of this strain are glycine, proline, hydroxyproline, alanine, glutamic acid, aspartic acid and serine, which together account for over 60% of the total protein nitrogen of collagen, the main product of the mechanocyte (see p. 52). Cystine has been found to be essential for the growth of mechanocytes from bone (osteoblasts) and from muscle (myoblasts) and it could not be replaced by methionine (Fischer, 1948), as it can for some purposes in the body, though its action is assisted by methionine (Morgan and Morton, 1955). In tissue cultures, lysine is not necessary for the growth of myoblasts (mechanocytes from muscle), but does benefit the growth of osteoblasts (mechanocytes from bone) (Fischer, 1948): this is interesting because lysine is, of course, essential for the growth of the whole animal. Once again, this is further evidence that the cells which masquerade in tissue cultures as mechanocytes are not all identical, but that there are metabolic differences between them, and that they exist in certain fairly well-defined races. Glutamine does not appear to be essential for chick heart fibroblasts (Fischer, 1948; Pasieka, Morton and Morgan, 1956) but is required by periosteal fibroblasts, and is required by the L-strain of mouse fibroblasts (Eagle, Oyama, Levy, Horton and Fleischman, 1955).

THE RACES OF MECHANOCYTES

It is thus probably true to say that although by prolonged cultivation *in vitro* one may obtain a pure culture of mechanocytes to all visible appearances, yet such a so-called pure strain may well be a mixture of mechanocytes of several kinds. Moreover, as pointed out earlier, it may have acquired certain new characteristics by adaptation to the conditions of tissue culture. This idea of a mixture of types of mechanocytes can be verified to some extent by studying the nature of the tissue developed when cultures are allowed to redifferentiate once more after a period of rapid proliferation, i.e. of unorganized growth. The results with osteoblast cultures and muscle cultures have already been partly described, and it was observed in the former case that such mechanocytes have the power to differentiate as one would expect them to do, into a bone-forming tissue. Nevertheless, in a small number of cases, the differentiating cells from pure cultures of endosteal tissue produce cartilage and not bone (Fell, 1933) (Fig. 3.6). Either one must

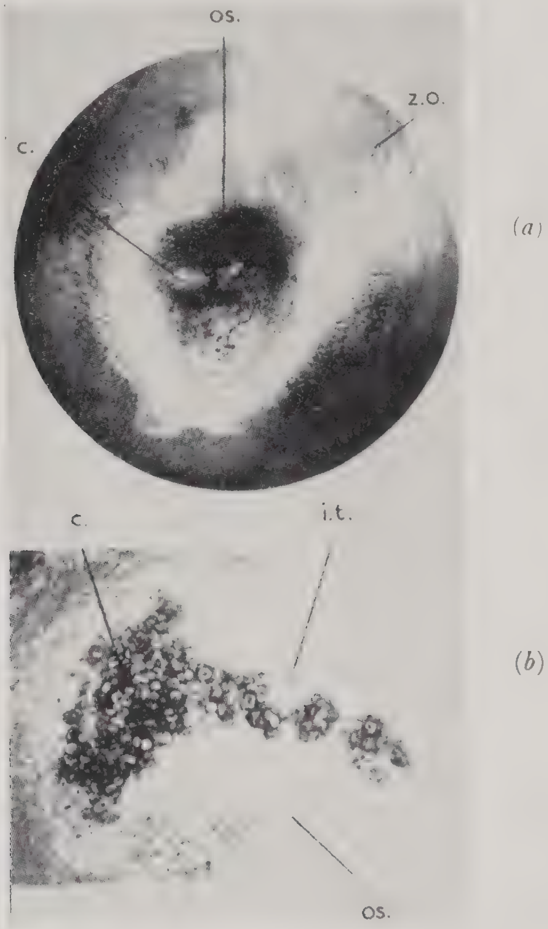

Fig. 3.6

Culture, originally derived from endosteal bone, showing the development of cartilage (c) as well as of bone (os) when encouraged to redifferentiate after a period of unorganized growth. (*a*) Living culture showing cartilage nodule. (*b*) Fixed and stained section through the culture. (Fell, 1933.)

assume that cartilage cells (chondroblasts) or undifferentiated "reserve" cells were somehow enclosed in the original explant, though since endosteal bone from the centre of the diaphysis was used, this does not seem very likely; or, alternatively, mechanocytes derived from bone (osteoblasts), still have the power, should the conditions evoke it, to produce cartilage instead of bone, i.e. to become chondroblasts.

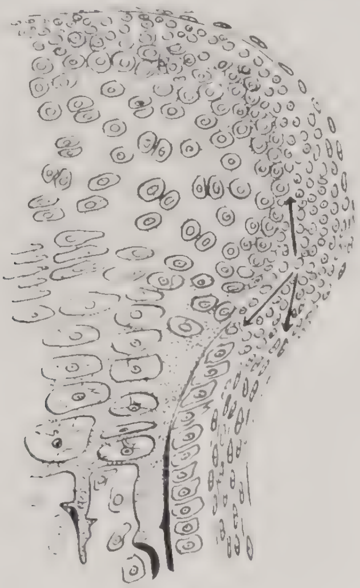

FIG. 3.7
A diagrammatic section on the margin of the epiphysis of a long bone showing the differentiation of primitive mesenchyme cells into chondroblasts, osteoblasts or fibroblasts.

As a visual example of the sort of differentiation involved in these cultures, attention may be called to the fact that if the lateral surface of the epiphysis of a developing long-bone is examined in histological section, there will generally be found one region where the cells are histologically more or less undifferentiated (Fig. 3.7). A small movement of the section in one direction shows these cells developing into indubitable cartilage cells, or chondroblasts busy with the formation of hyaline cartilage. A movement in the opposite direction shows a series of almost imperceptible changes, cell by cell, from the undifferentiated cells at the starting point to well-defined periosteal osteoblasts, which lie along the surface of the hypertrophic cartilage and are responsible for the formation of the developing bony diaphysis. If the field of view is now shifted once more from the osteoblasts of the periosteum to the fibrous layer of this membrane it will be found that, if this layer is followed towards the epiphysis and the point of origin, the fibrocytes of the periosteum grade steadily into the indifferent or undifferentiated cells once more. This particular zone on the surface of the epiphysis thus illustrates very clearly three potentialities of morphological development for the primitive mesenchymal cell which is probably in this case very much the equivalent of the tissue-culture mechanocyte, namely towards chondroblast, osteoblast or fibroblast. Cells from the synovial membranes (synovioblasts) can probably also be included in the same group as they behave very similarly *in vitro* (Vaubel, 1933a, b). Odontoblasts, the dentine-forming cells of teeth, are also probably similar, since they closely resemble osteoblasts *in vitro*.

These four types of behaviour (i.e. bone formation, cartilage formation, fibre formation and synovial membrane formation) are also four of the most easily demonstrated potentialities of one class of mechanocytes as seen in tissue culture and, as we shall see, other mechanocytes have other potentialities. The particular type of differentiation which is followed thus appears to be determined, at least partly, by conditions external to the cell. When cultures of osteoblasts are treated with embryo juice, they grow rapidly and the only sign of differentiation is that they produce argyrophil fibres, but in less growth-promoting media such cultures produce true collagen fibres and osteoid tissue, and, in a few cases, cartilage. This little sub-family of cells including true "fibroblasts," chondroblasts, osteoblasts, synovioblasts and probably a few others, e.g. odontoblasts, is thus characterized by its capacity to produce and to lay down between the cells varying proportions of collagen-type proteins and also mucoproteins, mostly containing chondroitin sulphuric acid, and sometimes to produce the enzyme phosphatase, which then, in a suitable medium, determines the deposition of calcium salts.

How far the different types of activity are mutually inter-changeable is uncertain and probably there may be steps in the process of differentiation which demand rather special conditions. For example, if the limb-bud from a 3-day old chick embryo is cultured on the surface of a plasma coagulum, the limb develops for a time as a whole and a cartilaginous skeleton appears within it, much as it might do in the intact animal. Such a skeleton, however, reaches a certain stage of development but does not develop further; no phosphatase appears in the cells; no hypertrophy of the cartilage cells takes place and there is no true ossification. On the other hand, if the original tissue explanted is the limb-bud of the 6-day embryo, then it continues to develop more completely; in it the cartilage hypertrophies and calcified bone appears in the usual places (Fell and Robinson, 1929). Obviously some conditions are established within the tissue, between the third and sixth days, which eventually allow the development of phosphatase by the relevant cells, and the full differentiation of the osteoblast.

A somewhat similar situation arises when two developing ribs are planted in a tissue culture at right angles to each other in the form of a T (Glucksmann, 1939, 1942). As the ribs continue their growth and development, one presses against the other and bends it. Under these conditions the morphology of the bent bone changes so that bone appears mostly where tension is highest, namely on the outer surface of the curved bone, while cartilage tends to appear from the perichondrium, and even the periosteum, on the lesser curvature where the cells are under compression.

Another particularly striking example of the effects of external conditions on the activities of mechanocytes is seen in the results obtained by Fell and Mellanby (1950, 1952) on the growth of developing long-bones in media containing a large amount of vitamin A in the form of the alcohol or acetate. In these cultures the cartilage cells not only laid down no matrix but they actually resolved that which they had already produced, and gradually the cartilage disappeared as such and the still healthy cells became lost among, and indistinguishable from, the normal connective tissue cells. This change in the matrix probably involved the mucoprotein part rather than the collagenous part, for the basophilia and metachromasia disappeared, while the affinity for Van Gieson's stain was enhanced. In similar experiments on the ossifying bones from the mouse, there was also destruction of bone matrix caused by the excess vitamin A, but in the chick this was not so evident.

Myxoblasts and Myoblasts

All these observations point to the close functional relationship between those mechanocytes which can be classed as osteoblasts, chondroblasts, fibroblasts and synovioblasts. On the other hand, there is no suggestion that osteoblasts or their relatives ever become heart muscle cells, plain muscle cells, or skeletal muscle cells which are all cells which can also behave *in vitro* as mechanocytes. Nevertheless, there are reasons for believing that these three types of muscle are themselves not unrelated. There are records of plain muscle, as in the bladder of the dog, becoming striated when it is made to contract more frequently than normal. In tissue cultures of skeletal muscle the myoblasts which separate from the muscle behave for a time very similarly to the separate cardiac muscle fibres and may develop very similar patterns of longitudinal striations or "tension striae". The cross-striations are not an essential part of the basic contractile process and all

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three types of muscle may contract without any visible striations (Lewis and Lewis, 1917, 1924; de Rényi and Hogue, 1934). On the other hand, under conditions of tension all may develop "tension-striae" within their cytoplasm. Biochemically their metabolisms do not seem to be very dissimilar, though the organization of the tissues and the time relationships of contraction may be very different.

It would seem justifiable therefore to suggest that the main family of mechanocytes as classified by their type of growth and general behaviour in tissue culture may be subdivided into at least two subfamilies which could be conveniently called myoblasts or potential muscle cells, and myxoblasts or cells potentially concerned with the formation of skeletal and supporting elements. This term "myxoblast" refers to the outstanding property shared by fibroblasts, chondroblasts, synovial cells, osteoblasts and odontoblasts of producing and liberating into their surroundings varying amounts of mucoprotein (μύξα (Gr.) = mucus), which may be contrasted with the similarly well-developed property of the myoblasts which is to form within themselves the contractile protein acto-myosin. This does not mean that myoblasts cannot produce mucoproteins nor that myxoblasts are unable to form actomyosin, but simply that in each case these properties are not well developed. Collagen production, one of the primary and most typical characters of mechanocytes, is on the whole better developed in the myxoblasts than in the myoblasts, but it is certainly not their exclusive property. Prolonged cultivation of muscle in conditions not favouring growth has produced, as noted above, cultures containing little muscle and much collagen; and cardiac tissue has produced argyrophil fibres *in vitro* (Bloom, 1930). The origin of this collagen is, of course, not certain —it may have been produced by the interstitial connective tissue cells of the original explant, or it may genuinely have been developed by the dedifferentiated muscle cells. This, incidentally, illustrates one of the main difficulties of the tissue culture method, namely the correct identification of cell types and the origin of the various cells and structures which develop. Tissues as such are seldom, if ever, pure, and it is all too easy for an apparently uniform culture of cells to contain small numbers of cells of other types and for these to escape notice.

Collagen-type proteins, which are essentially chains containing a high proportion of glycine, proline and hydroxy-proline molecules having a distinctive X-ray diffraction pattern, are characteristic of many of the tissues from which mechanocytes emerge in tissue culture. These tissues are all derivatives of the loose mesenchyme, the majority of whose cells are virtually synonymous with the myxoblasts. Collagens are not normally produced in any appreciable amount by any of the essentially epithelial tissues, glands or strictly neural elements in the body. From the work of Gross and others (Gross, 1956; Gross, Highberger and Schmitt, 1954) it appears that collagen results from the building up of "tropocollagen" units in a rather special manner into fibres (Fig. 3.8). Tropocollagen units are stiff rods, about 2900 Å long and 14Å wide, and having a "molecular" weight of about 340,000. The tropocollagen units or their immediate precursors are liberated from the cells more or less in proportion to the growth rate of the tissue and appear in the surrounding medium where, given the right conditions, they line up in a staggered pattern to produce cross-banded fibres with a spacing of 640Å (Fig. 3.9). Although it is possible in the test tube to produce the 640Å banding of natural collagen fibres from tropocollagen units the actual conditions for their formation in the body are not yet known. Mucopolysaccharides and vitamin C appear to be involved *in vivo* in argyrophil fibre formation, but, so far, it has not been shown how they contribute biochemically.

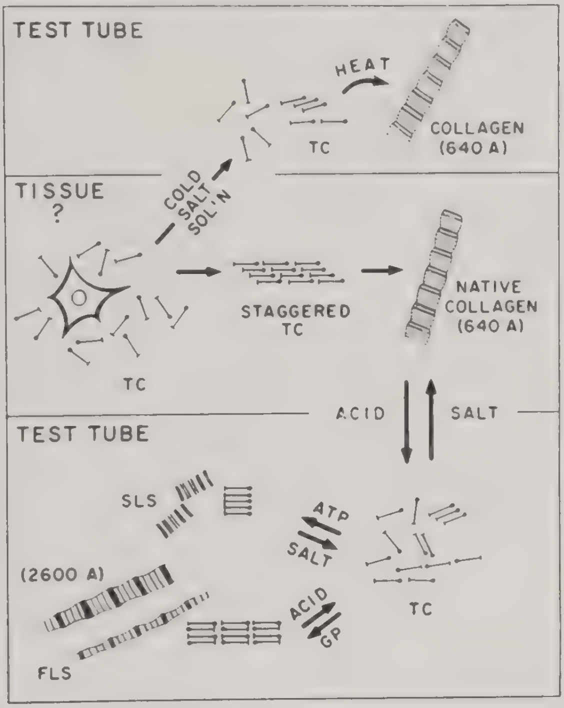

FIG. 3.8

Scheme for the building of collagen fibres from tropocollagen units, both artificially and naturally.

ATP—Adenosine triphosphoric acid. FLS—Fibrous long spacing.
GP—Glycoprotein. SLS—Segment long spacing. TC—Tropocollagen.
(Gross, 1956.)

Apart from collagen formation on the one hand and contractility on the other as distinguishing features, the importance of lysine for osteoblasts and not for myoblasts, as mentioned earlier, may perhaps point to a further biochemical difference between the two sub-families, myxoblasts and myoblasts.

For various reasons, neither osteoblasts nor chondroblasts tolerate high concentrations (i.e. more than 15% of embryo juice) for long, though the mitotic rate in osteoblasts may be increased temporarily by concentrations up to 40%: heart and muscle mechanocytes, on the other hand, are not so adversely affected by high concentrations of juice nor are their mitotic rates much increased by concentrations between 15% and 40%. All these observations taken together support the concept of a division of mechanocytes into two main families, in each of which there may prove to be many genera and species.

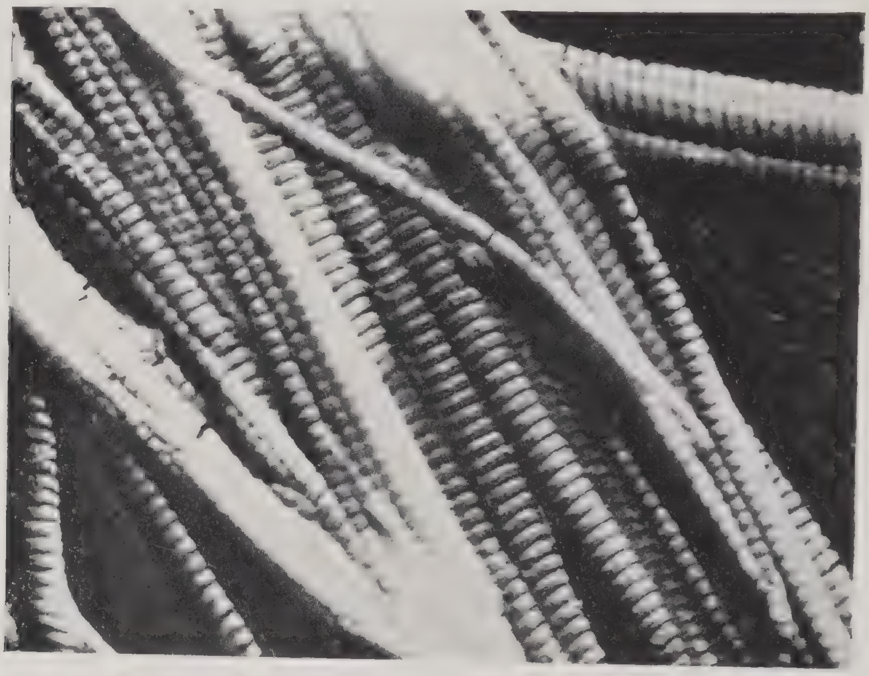

FIG. 3.9
Collagen fibres showing the typical cross-banding. Electron micrograph of leg-tendon of fowl. (Magnification × 31,000.) (Randall, 1953.)

Before leaving mechanocytes to consider other families of cells, something more may perhaps be said about the endothelium of blood vessels especially as it emerges from cultures of the heart and liver. From cultures of fresh heart tissue the endothelial cells often emerge as a continuous sheet of polygonal cells. Such cells from the heart *in vitro* often show tension-striae (Lewis and Lewis, 1924) and endothelial cells *in vivo* are mildly contractile. Their typically membranous growth of extended and flattened cells easily breaks up and gives rise to separate cells not unlike fibroblasts; in fact, Lewis (1922, 1923) has described all stages in the transformation. The simplest hypothesis would therefore be that these endothelial membranes represent the reaction of myoblasts, which are normally present in the mesenchyme, to growth on a surface, though the actual conditions which evoke the epithelioid type of behaviour have not yet been worked out. Such endothelia (and they are the majority of those in the body) are probably to be distinguished from a few special endothelia derived from such situations as bone marrow, spleen, liver sinusoids and adrenal cortex, where cells of a different type may be involved (see p. 91) and are distinguishable by their well developed phagocytic properties. If this flattening of the cells is the reaction of myoblasts to contact with a surface, then the synovial membranes (King, 1935) (Fig. 3.10) and the layer of odontoblasts (Fig. 3.11) in teeth may well represent the manner of behaviour of myxoblasts under similar conditions. In these two cases the cells tend to take up a much more columnar shape and the whole membrane resembles a somewhat loose columnar epithelium rather more than a pavement. It should, however, be stated that on the surfaces provided in tissue cultures, synovioblasts may also produce a membrane-like growth of flattened cells (Vaubel, 1933a).

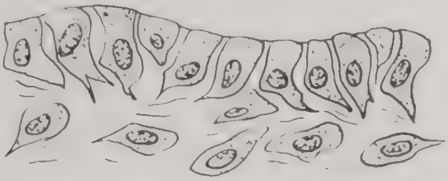

FIG. 3.10
Synovioblasts showing the semicolumnar arrangement which these cells sometimes assume. (Re-drawn from King, 1935.)

From all that has been said so far it seems possible to conclude that there is a large group of tissues in the animal body which, when explanted *in vitro*, give rise to cells which display mechanocytic behaviour; this behaviour is undoubtedly a simplification of normal cell function, but is only partly a dedifferentiation. Individual potentialities may still persist more or less irreversibly and can be evoked again by suitable conditions. Within the main family of mechanocytes there is the suggestion of at least two subfamilies, myoblasts and myxoblasts, and, pushing this idea of classification further, the myoblasts could be divided into genera of cardio-myoblasts, skeleto-myoblasts, viscero-myoblasts and perhaps others like "endothelio-myoblasts," while the myxoblasts would fall mainly into three rather closely related genera of osteoblasts, chondroblasts and fibroblasts and the more aberrant synovioblasts and odontoblasts. It must be remembered in using this analogy with the classification of species, that unlike the latter, it is not based on quite such an irreversible system.

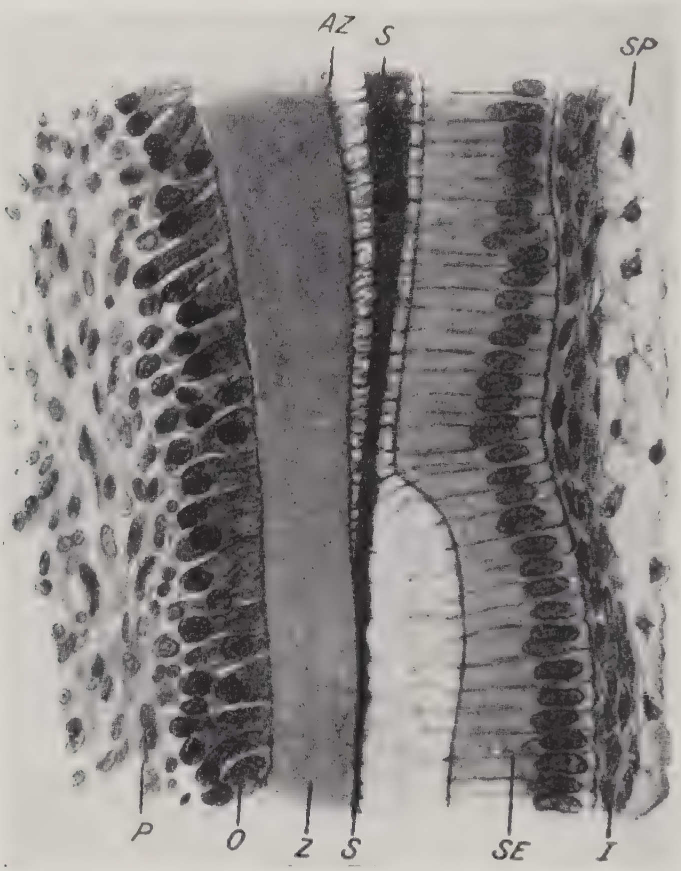

FIG. 3.14

Diagram showing the pseudocolumnar arrangement of odontoblasts (O). P—Pulp. Z—Dentine. S—Enamel. AZ—Dentino-enamel junction. SE—Ameloblasts. I—Flattened cells of enamel organ. SP—Stellate cells of enamel organ. (Maximow and Bloom, 1930.)

In other words, while species cannot change from one into the other, though the boundaries between them may sometimes be ill-defined, cells by reversing their evolution appear to be able, to some extent, to start again on another line; this at least follows if the appearance of cartilage in pure cultures from endosteal bone as described above has been correctly interpreted. However this may be, a question which must be asked is: "how do myxoblasts in tissue culture continue to produce myxoblasts, and myoblasts to reproduce myoblasts?" As we shall see, mechanocytes are recognizably different from other cell types *in vitro*, but after prolonged periods of continuous sub-cultivation, in a medium of plasma and embryo-tissue juice, cultures of mechanocytes *in vitro* have remained essentially the same as they were at the end of the first passage. They have bred true as mechanocytes. It should be stressed, however, that under other conditions of cultivation, generally involving more complete isolation, mechanocytes have acquired new characteristics *in vitro* and have adapted themselves to the new medium and conditions, and become "tissue-culture-cells".

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CHAPTER 4

THE GROWTH OF EPITHELIOCYTES

When tissue cultures are made from the skin or from the intestine, in addition to the growth of mechanocytes which emerge freely from the dermis or from the muscle coats, and from any connective tissue present, there also appear, sooner or later, cells which behave quite differently. These are the cells of the epithelium, and they emerge from the cut edges and spread out on any available surface in a single layer, as a continuous sheet of more or less flattened cells, i.e. as a closely packed pavement epithelium (Fig. 4.1). In this behaviour the epithelial cells are doing exactly what they may do in the body, as for example, during the first stages in the repair of a wound in the skin. When a portion of skin is removed, the more basal cells of the epidermis

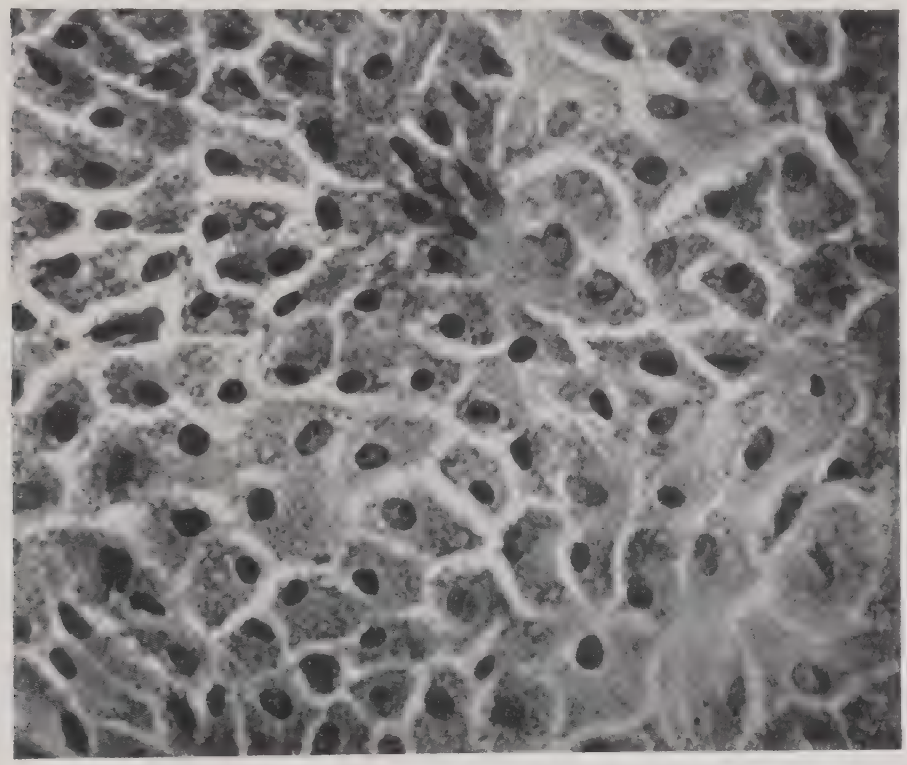


FIG. 4.1

Growth of epithelium from the iris of the chick embryo. (Fischer, 1922.)

all around the cut very soon flatten out and migrate centripetally over the damaged surface, provided that the latter is in a condition suitable for their activity. In doing this the cells undergo few, if any, divisions; they simply tend to spread as a continuous sheet over the wound; at first, this sheet is only one layer thick, and the cells are all closely adherent to each other; though they may change their relative positions the cells remain as a membrane. Only later do cell divisions occur round the margins of the wound and then eventually spread centrally and assist in thickening up the membrane originally formed by cell migration. Cells behaving in this cohesive way have been observed in cultures of skin, intestine, liver, pancreas, kidney, pigment epithelium in the eye, thyroid and many other tissues, but always from those tissues which originally contained cells of true epithelial type i.e. cells which formed part of the primary surface of the animal. Thus epithelial cells from many, if not all, true epithelia are found to produce similar membranous sheets of cells in tissue cultures, always provided that a suitable surface is present upon which they may creep. In contrast to the behaviour of mechanocytes, where the cells normally emerge as a network, it is highly characteristic of these cells of epithelia that they maintain close connections with each other all along their edges of contact and no spaces normally develop between them. There may be a 'cement substance' or at least a specialized region of the cell surface between the cells which becomes black when treated with silver nitrate. This, however, is not a diagnostic feature since it occurs between endothelial cells also, which, as discussed in the last chapter, are probably related to mechanocytes. There may even be fibrous or pseudo-fibrous structures running between cell and cell in much the same way as they appear to do in the prickle-cell layer of the human epidermis. In spite of their close contact, however, the cells remain as discrete entities, and damage to or death of one cell does not involve changes, except of a mechanical nature, in the adjacent cells, as it would do if the cells were actually connected by protoplasmic bridges. Chambers (1924), for example, has shown that so long as the daughter cells after mitosis are still connected by a protoplasmic thread, then puncture of the nucleus of one of the cells causes coagulation of the cytoplasm of both cells, but similar damage to single cells in an epithelium is not transmitted to neighbouring cells.

Although superficially similar, true epithelial growth can usually be distinguished from the epithelioid growth of most vascular endothelia. The cells of the latter are generally larger and appear to have a somewhat different internal organization. However, the nature of the relationship, if any, between the cells of the endothelia of blood vessels, the cells of the so-called mesothelia (e.g. serous membranes of pericardium and peritoneum) and the cells of the epithelia discussed here is obviously a matter of importance, and at present is anything but clear. Further evidence, extending beyond the more purely morphological, is urgently needed as to their specific properties, potentialities and general behaviour, and the chapters which follow may suggest lines of investigation.

As emphasized already, the form of growth described above for the true epithelia is naturally dependent upon the provision of a suitable surface along which the membrane of cells may extend. This is normally, in tissue culture, provided by the glass surface of the coverslip, flask, or culture vessel, or alternatively by the surface of a plasma clot, or even an agar gel. The surface of the plasma clot initially forms an excellent basis along which the cells readily extend, but sometimes the cells may liquefy the plasma and then the whole membranous structure may break down, or the epithelium may roll up into tubular structures and irregular masses. In any case, the stretched membrane is always a rather unstable structure and epithelial cultures are very apt to disintegrate into separate nodules or rolls of tissue. The membrane may quite suddenly break, as if it had been under considerable tension; it then rolls up on itself, and this rather tiresome property from the point of view of tissue culture has made the detailed analysis of the growth in these membranes lag behind that of the growth of cells of other types, for the necessarily long-continued observations on a small group of cells under various conditions is almost impossible. On the other hand, when epithelial cells pile up in this way and do not persist as a flattened sheet, there is obviously more scope for them to form tubular or glandular structures, and this they often tend to do under these conditions.

When epithelial cells are buried in the substance of the plasma clot the growth is much more restricted and in this situation, tongue-like and tubular structures often appear (Fig. 4.2), the latter probably following the cavities produced by liquefaction of the coagulum. With some epithelia there is, under such conditions, a tendency for isolated cells to break away from the membrane and to adopt a more mechanocyte-like type of activity. Here again more observations and analysis are necessary. Are the cells, which thus break away, true mechanocytes or only mechanocyte-like in appearance? Reasons will be advanced on a later page for the belief that the break-up of an epithelium into separate cells, which may resemble mechanocytes or cells of other types, may be an inherent and latent property of epithelia in general.

As already outlined, successful growth of epithelial cells has been obtained from such widely different sources as the pigment epithelium of the iris and retina, lens, skin, thyroid gland, liver, kidney, intestine, pancreas and uterus. In each case the growth takes the usual form of a continuous membrane of cells, and is naturally favoured when the culture is placed on the surface of the coagulum bathed with sufficient fluid to keep the cells in a moist environment. It is clear from a study of this list that it is of no significance whether the original tissue contained a stratified, glandular or any other sort of epithelium, whether it belonged to an endocrine gland or an exocrine gland, or whether it was derived from ectoderm (e.g. skin), endoderm (e.g. intestine) or mesoderm (e.g. kidney). The general form and pattern of growth *in vitro* is essentially the same, and fundamentally different from that of the growth of mechanocytes.

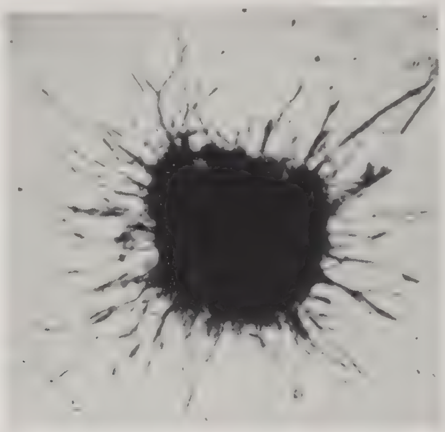


FIG. 4.2

Tubular and cord-like growths of epithelium from the iris when growing in a solid coagulum. (Fischer, 1924.)

Once again, as in the study of mechanocytes, the mechanical qualities of the medium and substratum have their effects in determining cell morphology, and the findings on the behaviour of epithelia in culture would have little significance if they rested only on morphological characteristics. Although the tendency of the cells to stick together so as to form a continuous membrane, a tubular structure or cords of cells is extremely characteristic of the epitheliocyte type of growth, yet these cells may not always display this behaviour, and it has already been noted that at the edge of the growth zone of epithelia there are often to be found cells breaking way from it and assuming a more independent mode of existence. The classification of such cells on their appearance alone may, as with mechanocytes, be a difficult task, but once again their movement as seen in cine-recordings is sometimes diagnostic.

The Products of Epitheliocytes

When cells from various epithelia are kept in culture, there is often a tendency for them to undergo changes which are almost certainly connected with the process of keratinization (Fischer, 1924; Miszurski, 1937), i.e. the formation of the particular type of sclero-protein known as a keratin (Fig. 4.3). This of course is the class to which the natural protein-product of the epidermis in man and indeed of most vertebrates belongs. It is a type of protein with a characteristic X-ray diffraction pattern which is significantly different from that of the corresponding protein-product of mechanocytes, namely collagen. The amino-acid constitution of keratins is also quite different, and much of their structural character depends upon the cross-linking of the peptide chains by sulphur bonds. The protein arises within, and remains far more intimately associated with, the epithelial cells than does collagen with the mechanocytes, for the latter is certainly liberated into, if not actually formed in, the surrounding medium. Keratinization generally occurs within the cytoplasm, being preceded by an increased concentration of SH groups in the cells, and it eventually involves the death of the cell. In tissue cultures, cells which are undergoing these changes generally become granular and then vacuolated in a characteristic manner. In the body, keratinization is normally confined to the epidermis and its immediate derivatives, like the hair follicles and the enamel organs of the teeth. It also occurs periodically in the vagina. In conditions of severe deficiency of vitamin A, other epithelia may assume this activity, e.g. the conjunctival epithelium,

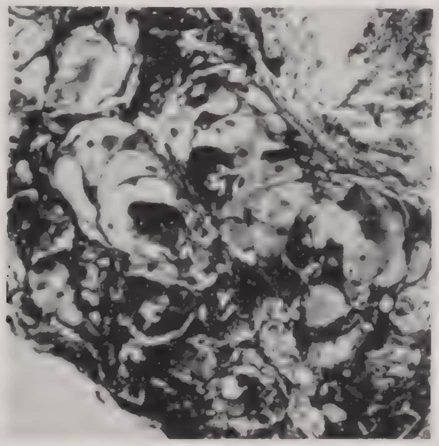


Fig. 4.3

Cell of iris epithelium undergoing changes resembling keratinization. (Fischer, 1924) and the tracheal epithelium; in malignant disease, keratinous or prekeratinous changes may be found in a variety of other epithelial structures. Some birds have keratin in the gizzard and some have hair-like processes which guard the entry to the pylorus. *In vitro*, keratinization has been observed in a variety of other epithelia, e.g. the pigment epithelium behind the iris, and it seems justifiable to conclude that keratin formation is about as typical of epithelia as collagen formation is typical of mechanocytes. It seems to be a latent potentiality of epithelia, even when it does not normally manifest itself.

Another characteristic of epithelial behaviour, although not one which is often observed in tissue cultures except under such conditions as lead to the differentiation of cells, is the formation of mucoproteins and mucopolysaccharides. This, of course, was also true of mechanocytes, but while mechanocytes produce these substances in the form of a matrix between the cells, many epitheliocytes more conspicuously produce them as droplets within the cytoplasm. Typically the secretions of the mechanocytes contain chondroitin sulphuric acid, containing galactosamine, while the corresponding sulphated epithelial mucin (mucoitin sulphuric acid), which may of course also be finally liberated from the cells, more often contains glucosamine in place of the galactosamine. It would be interesting to know whether the stereochemical difference between the sugars is in any way related to the location of the product with respect to the cell membrane. In lower animals, chitin, as a glucosamine compound, is also interesting as being an essentially epithelial product, and the chemical properties of the 'cuticles'', "terminal bars" and "cement substances" between the cells of the epithelial tissues of the vertebrates call for further investigation along these lines. It is perhaps significant that such structures tend to be more prominent between those epithelial cells which are not themselves engaged in "mucin" storage. On the other hand, the hyaluronic acid of the connective tissue "ground substance" is also a glucosamine derivative, so it appears from this that glucosamine may be liberated not only by epitheliocytes but by mechanocytes also, or at least by some cells in the connective tissue.

Both in mechanocytes and in epithelia the formation of mucin is affected by the vitamin A content of the medium but the results are different. In normally keratinizing epithelia, e.g. the skin of the chick, an excess of vitamin A in the medium turns many of the cells into mucus-producing cells (Fell and Mellanby, 1953) (Fig. 4.4); in the opposite direction, i.e. in cases of vitamin A deficiency, the mucous cells in the trachea *in vivo* may cease to function as such and begin to keratinize. In these epithelia then the presence of vitamin A in some way favours mucoprotein production and depresses keratinization. These effects contrast rather strongly with those already mentioned in connexion with mechanocytes, in which an excess of vitamin A when added to cultures of cartilage and bone, led to the complete disappearance of the mucoprotein matrix, presumably as the result of some new activity on the part of the mechanocytes, since it was found that these must remain present as healthy cells for the process to go on. The activity of an enzyme, forming soluble sulphated mucopolysaccharides in place of chondroitin sulphate has been suggested (Fell, Mellanby and Pelc, 1956). Recent work also indicates that the changes in the epithelial cells may be connected with the manner in which sulphur is picked up and metabolized by the cells. For example, Flesch (1953) has suggested that the action of vitamin A is to inactivate the SH groups in the cell. Moreover, autoradiographs obtained after treating tissues in culture with radioactive S show that mucus-secreting cells can pick up their sulphur in the inorganic form whereas keratinizing epithelia do not appear to be able to do so. Presumably they must get their sulphur in the form of cystine or methionine. Perhaps the mechanocytes have to get at least some of their sulphur in the same way for it has already been observed that cystine is an essential constituent of the medium for the growth of mechanocytes. The ability to use the inorganic sulphur may be connected with the movement of glucosamine or its presence intra- or extra-cellularly.

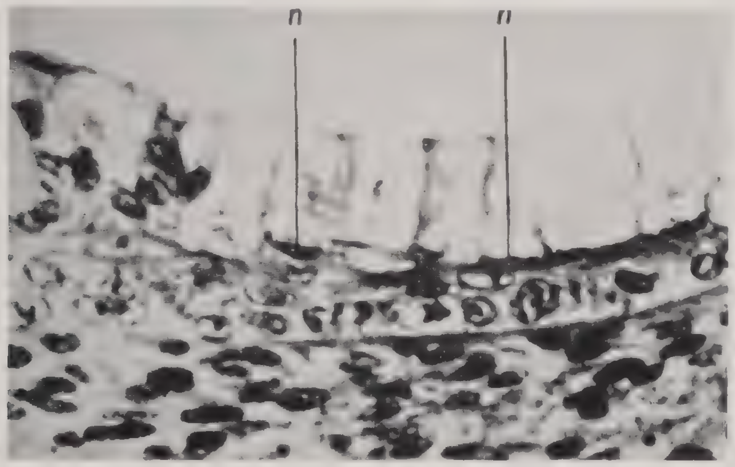


FIG. 4.4

Mucous cells in the skin of the chick produced by high doses of vitamin A in tissue cultures. n—nucleus of mucous cell. (Fell and Mellanby, 1953.)

With reference again to the "cement substance" between epithelial cells, it is interesting to notice that trypsin alone does not cause the separation of the cells of an epithelium as satisfactorily as it does those of the connective tissue when these tissues are similarly treated. On the other hand, when trypsin treatment is combined with Ca^{++} and Mg^{++} deficiency, epithelial tissues fall completely apart and this method is used for disintegrating embryonic tissues (Moscona, 1952 a and b; Rinaldini, 1958). The direct disintegrating action of Ca^{++}-free media on the cement substance between epithelial cells and the subsequent rounding up of the cells has been frequently observed in blastulae, endothelia of blood vessels and in tissue cultures of the skin. Moreover it is a matter of some interest to the present discussion to note that L-ascorbic acid is not necessary for the production of the intercellular substance by epithelial cells (Chambers and Cameron, 1943), though it is necessary if mechanocytes are to produce their intercellular ground substance properly as in teeth, bones, and repairing wounds. However, in the latter case the ascorbic acid is perhaps involved more with collagen fibre formation than it is with the mucoid material, though it is sometimes difficult to separate the early collagen from the carbohydrate or polysaccharide which usually surrounds it. Indeed the polysaccharides are thought by some to have an orientating action on the protein fibres though their action in this direction has been questioned by Gross (1956). It may be recalled that many marine blastulae and embryos in the early cleavage stages may be broken up into their constituent cells by shaking with Ca^{++}-free sea-water. The cells of such embryos and blastulae are in many ways comparable with those of an epithelium in that they are normally coherent and, at any rate in blastulae, extend as a single layer around the organism.

When isolated from each other and returned to a medium containing Ca^{++}, epithelial cells tend to adhere to each other and to aggregate. This was well shown by Holtfreter (1947) for the isolated ectodermal cells of amphibian larvae (Fig. 4.5). It is equally true of the flagellated epithelial cells (choanocytes) of sponges and of human epithelial cells both from the skin and from the liver and kidney. The calcium ion may therefore be regarded as essential for maintaining the adhesive quality of epithelial cells. Nevertheless, a low Ca^{++} concentration favours the growth of skin epithelia *in vitro*, and so does a high phosphate content (Parshley and Simms, 1950). This may perhaps be so because of the greater motility allowed to the cells by the decrease in rigidity of the intercellular substance; but it should be emphasized that the biological actions of the calcium ion are many and various. Electron-microscope studies of the boundaries between epithelial cells would not suggest that the mechanism of the adhesion between the cells is always identical; nevertheless as soon as artificially isolated epithelial cells find, in the course of their almost random movement, others of their own type, they remain mutually adherent and seldom leave each other again. Epithelial cells of two different origins, however, do not tend to adhere to each other, at any rate for so long or so permanently as those of similar origin. Liver cells recognize other liver cells, and epidermal cells cohere with epidermal cells, but liver cells and epidermal cells are soon divorced. Liver epithelia and epidermis, however, are both genuine epithelia whose cells grow as continuous sheets (Weiss, 1958).

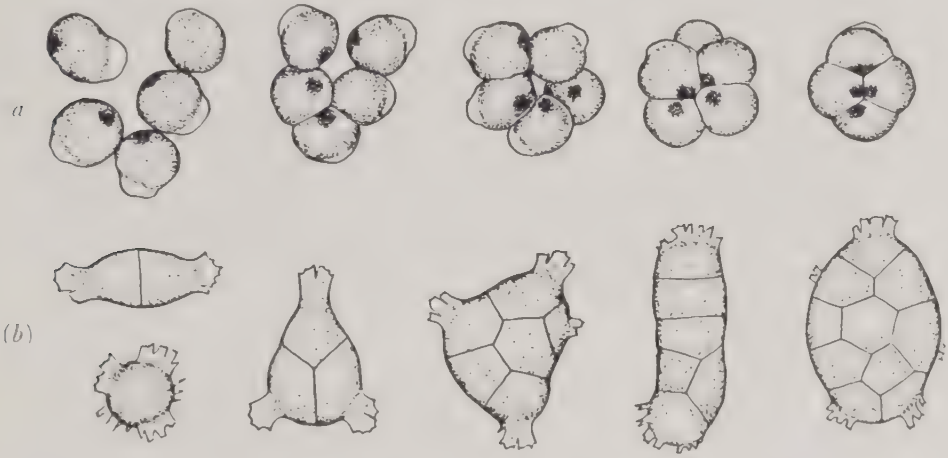


Fig. 4.5

(*a*) Aggregation of ectodermal cells after their isolation. (*b*) Ectodermal cells aggregating while creeping on a glass surface. (Holtfreter, 1947.)

Requirements for Growth

Unlike the growth of mechanocytes, the growth of epithelia does not appear to be so specifically dependent on embryo juice or extract, and successful growth of membranes of kidney, liver and intestinal epithelia can be obtained on the surface of clotted plasma alone. Indeed concentrations of embryo juice higher than 20% have been shown to inhibit the growth of membranes of hepatic epithelium (Doljanski, 1929). This point, however, as mentioned earlier, needs further investigation because there are practically no measurements of mitotic rates in cultures of epithelia in different media comparable with those carried out on mechanocytes. Many authors certainly use embryo juice in the medium for epithelia, and Doljanski (1930a), has found that pigment formation (i.e. a process dependent upon differentiation and function) is encouraged in iris epithelium in media washed with heparin plasma, while "growth" occurs faster in plasma with 30% extract, thus indicating that in this case the extract does actually stimulate the growth. Puck and his school (1957) have recently shown that clones of fibroblasts only grow successfully when embryo extract is present in the medium, while "epithelioid" cells grow well in its absence. All epithelial cultures, however, seem to benefit from good conditions of oxygenation and from a high glucose content of the medium, and the speed with which glycogen disappears from the cells in liver cultures, unless sugar is supplied in a concentration greater than 0.5%, suggests an active carbohydrate metabolism (Doljanski, 1930b; Gill, 1938). This is even greater under anaerobic conditions when the glycogen disappears very rapidly.

As with mechanocytes, it seems probable that the nitrogen requirements of epithelial cells may be partially satisfied by proteoses and peptones, for there are reports of epithelial growth being greatly enhanced by proteoses, e.g. such as those in Wittës peptone.

Several strains of cells derived from epithelial tissues, e.g. liver, conjunctiva and intestine have been grown on mixtures of amino-acids with vitamins, glucose, salts and a small addition of serum protein. Under these conditions they all required thirteen amino acids (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, tyrosine and valine), and tended to degenerate when any one of these was missing (Eagle, Oyama and Levy, 1957). The same amino-acids were found to be necessary for a strain of cells originally established from a culture of mouse fibroblasts (strain L) but these results have to be interpreted with caution since the fibroblast strain is known to have become malignant during its life *in vitro* and the cells are thus no longer to be considered as "normal" fibroblasts. This same criticism can be applied to the epithelial cell strains and it is again possible that they too have lost some of their original properties and perhaps acquired others in becoming "tissue-culture" cells, though morphologically they remained epithelial and reasonably "normal" in appearance.

On the whole, epithelia are somewhat more tolerant of changed conditions than are mechanocytes; for example, the malt extracts, which inhibit the growth of mechanocytes, leave the growth of epithelial cells unchecked (Medawar, 1937) (see Fig. 3.5). Heated embryo juice, while somewhat harmful to mechanocytes, allowed epithelial cultures from the liver to grow quite freely (Doljanski, 1929).

Differentiation of Epithelia

So far, the growth of epithelial cells has only been considered in its "unorganized form", as sheets or membranes, and it has been pointed out that many types of epithelia can grow in this way, and that they all look very similar, though speeded up cine-films do allow some differences in behaviour to be observed. Once again, however, the cells still retain something of their individual characteristics for they can be caused to redifferentiate when the proper conditions are provided. The apparent "de-differentiation" to the simple membrane is therefore, at least for a time, again only a superficial and morphological dedifferentiation. Thyroid (Ebeling, 1924, 1925) (Fig. 4.6), salivary gland (Grobstein, 1955a) (Fig. 4.7), intestine (Törö, 1933) (Fig. 4.8), and kidney epithelia (Grobstein, 1955b) have all been made to resume some at least of their original properties after being reduced to

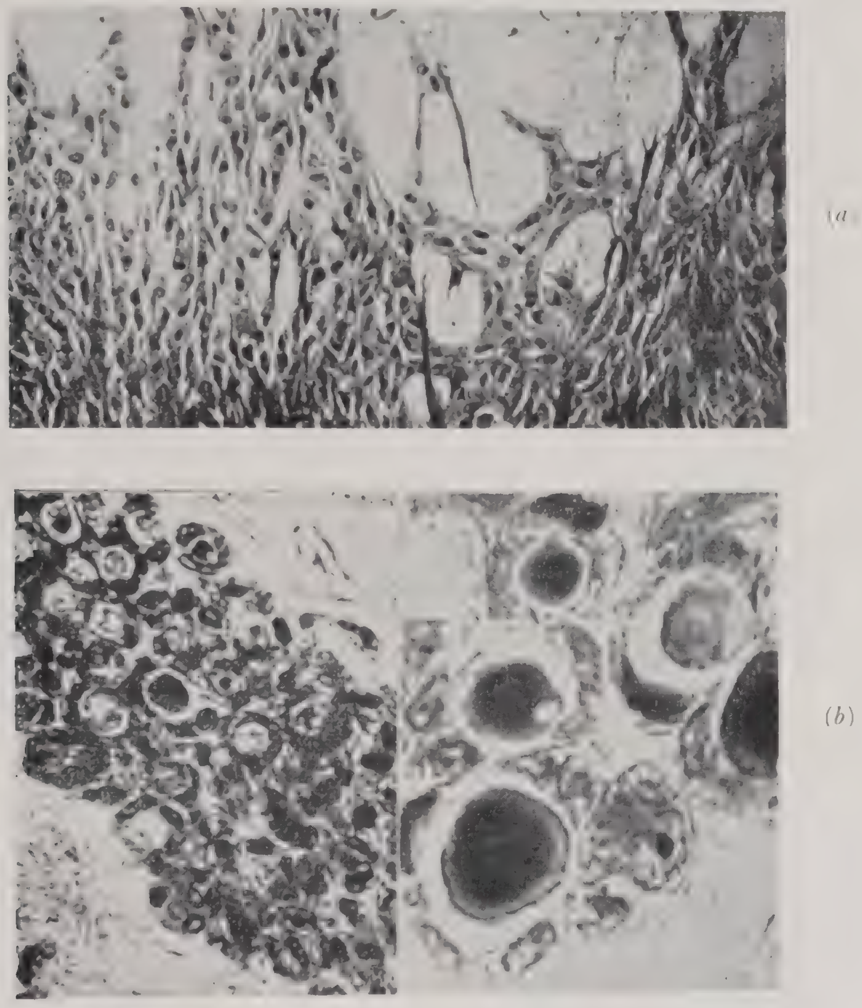


Fig. 4.6

(*a*) Epithelial cells from the thyroid gland in unorganized growth. (*b*) Sections of thyroid tissue redifferentiated in vitro. (Ebeling, 1925.)

simple membranes in tissue culture, and some of them after being grown in pure culture. Interestingly enough, a common factor in most—perhaps all—cases, in which the differentiation of the epithelium has occurred, has been the presence of mechanocytes. When an

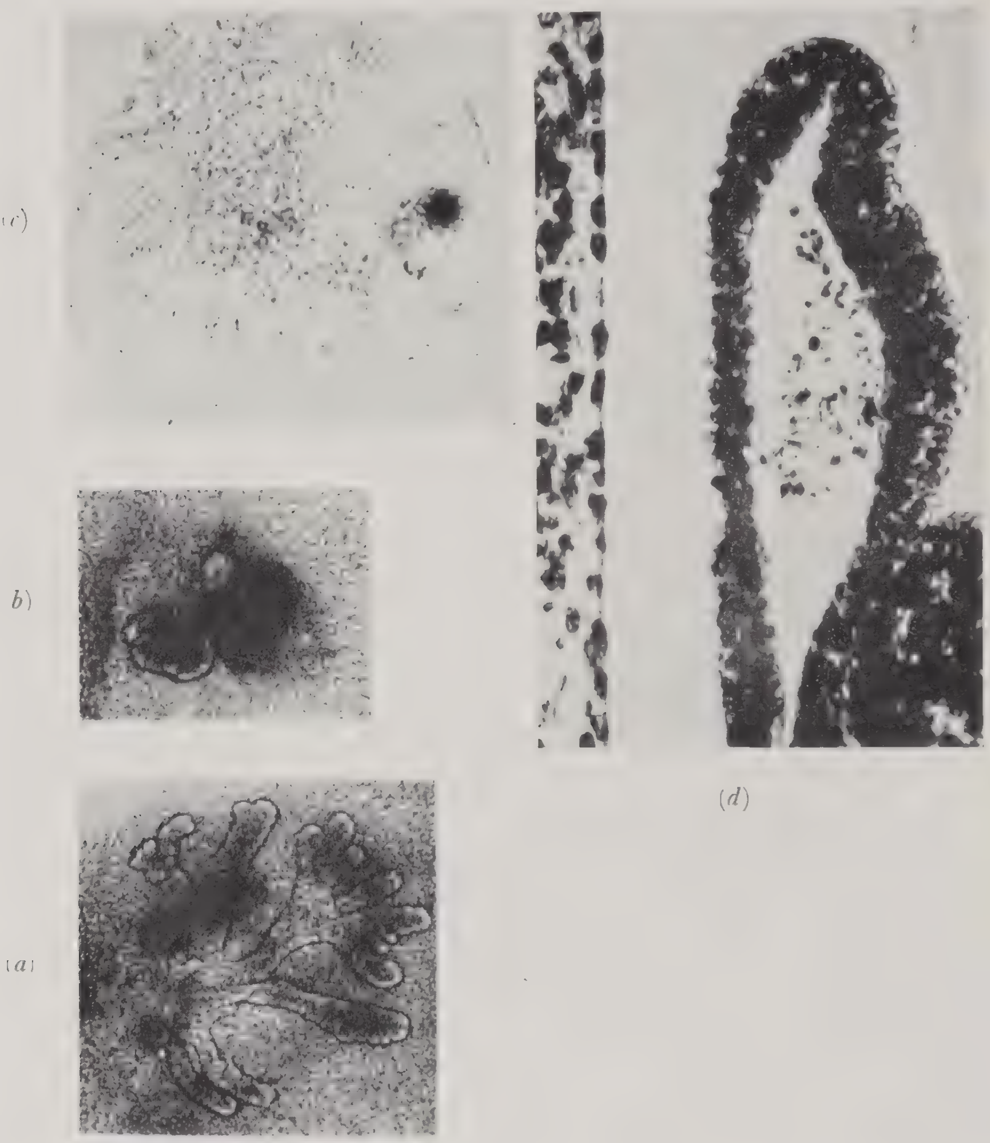


Fig. 4.7

Salivary gland epithelium developing into ducts and alveoli (*a*) after having been cultured as a simple membrane (*c*). The presence of autogenous mesenchyme is necessary for this differentiation. (*b*) Lack of differentiation with heterogenous mesenchyme; (*d*) Section of "tubule" on right of "millipore" membrane and inducing mesenchyme on left of membrane. (Grobstein, 1955a).

epithelium is kept in isolation no differentiation usually occurs. In the case of iris epithelium, however, the formation of pigment, which can perhaps be regarded as a function of the cells, is dependent only on the growth-rate of the cells (Doljanski, 1930a) and there are no necessary and accompanying morphological changes of any importance such as are necessary when most other epithelia start to function. The same can be said about the appearance of glycogen in pure cultures of liver epithelium when the growth rate is sufficiently reduced (Doljanski, 1930b).

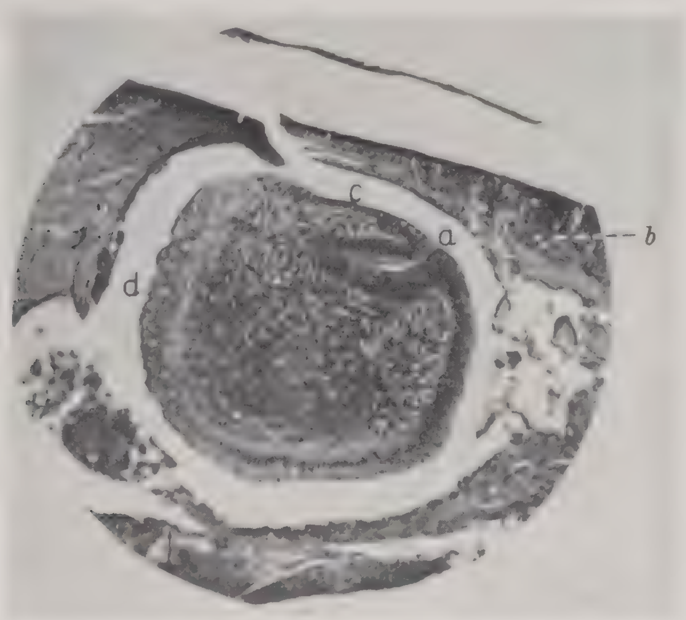


Fig. 4.8
Intestinal epithelium differentiating round a core of fibroblasts in the lens capsule of the eye after each had been grown as a pure culture in vitro. (Törö, 1933.)

When pure colonies of intestinal epithelium are grown with colonies of mechanocytes from the same source the epithelium tends to surround the mechanocytes and to differentiate into a columnar epithelium, sometimes with gland-like pockets (Törö 1933) (see Fig. 4.8). The iris epithelium, after growth in pure culture, has been found when mixed with a pure culture of fibroblasts to differentiate in the form of tubular and gland-like structures within the central mass of tissue, thus indicating not only the action of mechanocytes but also the versatility of epitheliocyte form (Fischer, 1930) (Fig. 4.9). Epithelium from the thyroid gland after many passages *in vitro* has also developed vesicles in the central mass of the tissue and produced secretion within the vesicles, particularly when the cultures were planted below the surface of the plasma clot (Ebeling, 1924) (see Fig. 4.6). It is not clear whether fibroblasts were present in these so-called pure cultures, but the illustrations suggest the presence of inter-acinar tissue. Kidney epithelium has produced tubules *in vitro* under similar conditions, i.e. when connective tissues were added to pure cultures of epithelium obtained by isolation of islands of kidney epithelium in the out-growth of tissue cultures by protecting them with mercury droplets while the rest of the culture was killed with ultra-violet light. Some reservation is necessary in the interpretation certainly of the last and perhaps of all these experiments, because of the general tendency of epithelia to form "tubes" in tissue cultures; but the peculiar character of the tubes produced is suggestive of true differentiation (Drew, 1923) and Grobstein (1955a and b) has shown the dependence of metanephric rudiments on

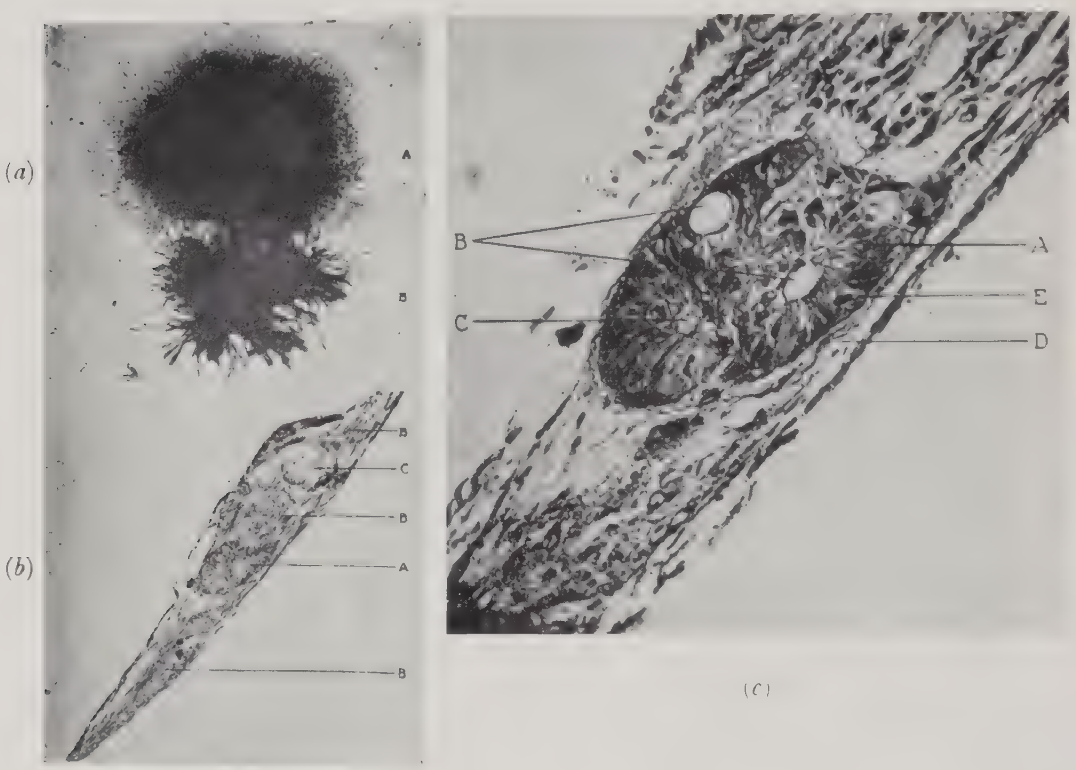


FIG. 4.9

Influence of fibroblasts on differentiation of epithelia. (*a*) Colonies of fibroblasts (A) and of iris epithelium (B) shortly after explantation. (*b*) Glandular structures of epithelium (A) embedded in fibrous tissue (B) after the cultures have fused together. Histological section of combined tissues. (*c*) High power view of epithelial growth in (*b*): A, glandular arrangement of cells; B, lumina; C, lumen filled with secretion; D, connective tissue fibres; E, nucleus of secreting cell.

(Ebeling and Fischer, 1922.)

other tissues for their differentiation and in this case mechanocytes are included among these other tissues. Moreover, when skin fragments (dermis and epidermis) are grown in a fluid medium in the presence of antibiotics, the epithelium (epidermis) quickly surrounds the dermal part and differentiates towards normal epidermis (Medawar, 1948). There thus seem to be fairly clear indications that epithelia of various sorts are caused or allowed to undergo histological differentiation by the presence of mechanocytes; whether these act mechanically, by orientation of intercellular "ground substance" or the like, through their secretions or their products of metabolism, or because they extract certain substances from the environment of the epithelial cells, remains for future research. Submandibular gland epithelium only undergoes differentiation when combined with certain specific types of mesenchyme tissue (Grobstein, 1953a and b), (see Fig. 4.7), and the indications are that mesenchymal cells produce their effects by the intervention of activities or "secretions" which may acquire rather specific qualities in some cases. There may even be two or more stages of action one to cause a check to membrance formation, others to cause actual differentiation. These effects on epitheliocytes *in vitro* may perhaps be compared with "induction" (or "evocation") and "organization" in embryonic development.

Mechanocytes may not be specific "evocators" in the sense that they cause completely indifferent cells to differentiate into a kidney epithelium or an intestinal epithelium, but they so contribute to the environment of epithelial cells that the latter can then display certain potentialities which may be latent within them or which they have acquired in their previous development (cf. Waddington, 1956). Kidney epithelial cells may grow as a continuous sheet of cells when provided only with a glass surface upon which to creep. In a plasma coagulum, or if allowed by some other means (e.g. by mechanocytes providing substances necessary for the formation of a basement membrane) to assume a three-dimensional pattern of growth, they may be able to develop tubular or other patterns. Mechanocytes may further contribute to the picture by so orientating the base upon which the epithelial cells creep and in other ways interacting with the epithelial cells, that the latter may subsequently be able to display their full potentialities. In such a system there are obviously various steps and stages, so that there is room for specificities to show themselves. Some mechanocytes, interacting with epitheliocytes, may be able to evoke the full pattern of development, as, for example, their own capsular mesenchyme can do with the epitheliocytes of the salivary gland rudiment; mechanocytes from other sources may only be able to evoke some aspects of the epithelial development but not the whole. Mechanocytes should not therefore be regarded as themselves inducers or evocators, but rather as contributors to that particular environmental situation for the epitheliocytes which allows them subsequently to develop their full potentialities. Evocators are thus not necessarily "substances" but may be "situations", and differentiation should be regarded as being dependent on the establishment of these local situations which may sometimes involve extremely complex and specific patterns. The molecular or structural orientation of the actively inducing agents may well be very important in addition to their physicochemical constitution. Moreover, the situations should not be regarded as static: almost certainly they are dynamic : gradients of concentration, and the flow of water, ions, and other constituents may be contributing to the behaviour patterns of the cells. Undoubtedly, however, the "proper" behaviour of many epithelial cells can, under these conditions, be allowed to develop by the presence of the proper mechanocytes. The problem, however, is certainly wider than this treatment perhaps indicates, for epithelial organization may sometimes be brought about by other epithelia of a different type and by their derivatives. For example, the metanephric rudiment has been caused to differentiate in culture by the presence of submandibular gland tissue in its vicinity and also by neural tissue from the dorsal part of the neural tube (Grobstein 1955b). Here again, probably these "foreign" cells contribute something to the environment of the metanephric rudiment which allows further development. The metanephric rudiment itself is, of course, not a simple epithelium and the environment of each of its constitutent cells is therefore already very complex. The whole question of these intercellular actions will need further discussion. The recent observations of Weiss, to which reference has already been made, show clearly that epithelial cells of various types have their own specificities, as is seen for example, when isolated epithelial cells from the epidermis recognize other epidermal cells and form associations and membranes with them, but ultimately always reject liver epithelial cells or kidney cells from such associations.

It is obvious from what has been said that epitheliocytes *in vitro*, although they may sometimes look very much alike in conditions of unorganized growth, still maintain some, at least, of the characteristics of their particular "species" or group, though often in latent form. Indications were given in connection with mechanocytes that this family of cells could be divided into sub-families, genera and even species of cells. The situation with epithelia appears to be somewhat similar, though we need to know more of the metabolic properties of the different types of epithelia before the classifications can be made on a very satisfactory basis. Moreover, since epithelia grow as continuous sheets, the presence of cells of different types may often escape notice because the methods which readily identify types of cells in isolation, e.g. the nature of their pseudopodia or their gait, are no longer quite so easy when applied to cells in a sheet. Puck *et al.* (1957) have suggested that the form assumed by cells is partly a "genetic" quality, since in the same medium, clones of mechanocytes and epitheliocytes continue to breed true, and that it is partly dependent on environmental factors, since the form of the cells can be varied in different media.

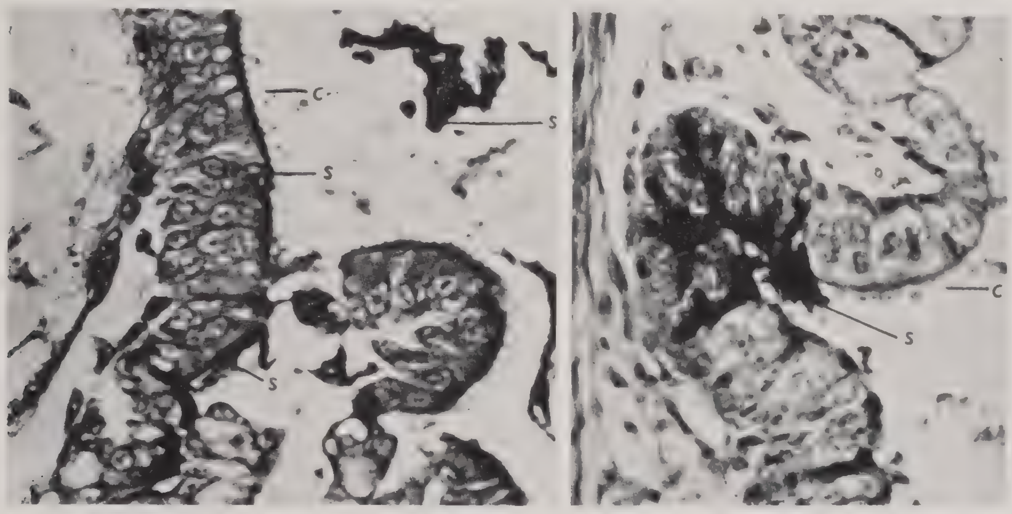
While it is abundantly clear that the various epithelia described, and indeed many others, produce *in vitro* a sheet-like type of outgrowth and that the cells have a natural tendency to adhere to each other, it has already been mentioned that there have been cases described in which the epithelial membrane tends to "fray" at its edges and for cells to leave it and assume a form resembling that of the mechanocytes, for example. Further information is, however, required on the external factors which cause this apparent transformation. We do not yet know how far such cells, which become isolated from epithelial sheets, acquire the physiological properties of mechanocytes, as for instance, collagen formation instead of keratin formation, inhibition by hexenolactone (which, it will be recalled, is probably the active substance in those malt extracts which differentiate between mechanocytes and epitheliocytes), or extra-cellular production of mucoproteins and other similar characteristics.

During the early stages of embryonic development all the cells of the organism are essentially epithelial cells in that they hold together as membranes. Sooner or later some of these cells acquire other properties and leave the primary layers to become mesenchyme etc. It is therefore possible that similar changes may occur when epithelial cells are cultured as membranes. Some of the cells may undergo this latent form of differentiation and become true mechanocytes. This is only a suggestion which might fit with the morphological change and as yet there is no evidence to prove or disprove the hypothesis. Incidentally, this tendency for the edge of the membranes to fray is more marked in some epithelia than in others.

If this change towards the mechanocyte form is a genuine differentiation, then it is probably more or less irreversible. Apart from the formation of the rather special endothelial membranes by mechanocytes, these cells certainly do not normally show any tendency to become epithelia with closely packed adherent cells. The change in the other direction must occur in embryology and may be the change which is occurring in the observations just described; it is at least worth bearing in mind as a possibility—though it must be remembered that the true change from epitheliocyte to mechanocyte is not only morphological but also involves radical metabolic reorganization.

Several epithelia (e.g. liver, conjunctiva and intestine) have now been grown as more or less pure strains in tissue culture, in fairly well-defined media and for long periods of time. Although they were certainly derived from epithelia in the first instance some of these strains have now lost the power to grow as sheets and they now multiply as colonies of individual cells. In some cases the cells are spindle shaped, in others they may have more or less membranous pseudopodia, like macrophages. The classification of these "products of tissue culture" certainly raises some interesting and important problems.

One point which has not been systematically followed up in tissue cultures, is the behaviour of the different types of epithelial cells from those glands or organs where there are originally several types of cells present. For example, it has been stated that in certain cultures of the adult kidney the cells of the convoluted tubules degenerated while those of the collecting tubules grew well (Nordmann, 1930). One would like to know if this was inevitable or did the particular culture medium and conditions of culture determine this selection. Would the tissue have behaved differently in another medium? In the normal intestinal epithelium there are goblet cells and ordinary columnar cells (excluding, the Paneth cells and argyrophil cells, which produce other secretions), but it is not known if these two types are detectably present in the membranes which grow from cultures of intestine *in vitro.* In the submandibular salivary glands there are mucous-secreting cells, serous cells and "demilune" cells; in the skin there are the ordinary squamous cells and there are dendritic cells. There does not, however, seem to be much reliable evidence as to how these different groups of cells behave *in vitro.* It would be most valuable to know whether they all assume the common epithelial type (which as shown above, is somewhat characteristic for each organ) or whether they have different metabolic properties persisting into the tissue-culture state. Putting the problem in another way, it has been shown that an excess of vitamin A converts a squamous epithelium (e.g. the skin of the chick) into one which indulges in mucin production. Is this a form of differentiation open to all epithelial cells given the right evoking agent, e.g. vitamin A? Or is it that certain cells can easily take this path while others cannot do so, or can only do so with great difficulty. Ciliated cells often appear among the mucous cells in such cultures (Fig. 4.10) and it is not clear whether this is a variant type of behaviour or whether there are always two types of cell in the epithelium, i.e. potential ciliated cells and potential mucous cells. In other words, are the ciliated and goblet cells of a normal trachea, for example, fundamentally different types of epithelial cells, or can one change into the other? In tissue cultures, ciliated and goblet cells are generally sloughed off before a stratified epithelium takes their place and *vice versa;* It would therefore seem to be unlikely that a ciliated cell can change into a goblet cell or that the reverse can happen. Both probably develop *de novo* from undifferentiated precursor cells, but it is not known whether the two types are derived from one uniform stock of basal cells or from two separate stocks which are not yet easily separable. The probability that there are two stocks is perhaps suggested by the fact that in those cultures of the chick's skin which were treated with excess of vitamin A, many of the cells became ciliated while others in the same culture became mucoid. We do not know, however, to what extent conditions may differ locally in such cultures and cause these differences in the behaviour of cells, so it is possible that the so-called basal cells may be able to differentiate along one of several lines according to conditions, i.e. to produce keratinizing cells on the one hand or to yield mucoid or ciliated cells on the other. Cultures of trachea have



(a) (b)

FIG. 4.10

(*a*) Epithelial cells of the skin of the chick which have become ciliated under the influence of high doses of vitamin A in tissue culture. (*b*) Normal nasal mucosa for comparison. c—ciliated cells. s—mucous secretion. (Fell and Mellanby, 1953.)

been made, in which unorganized growth occurred, but the question of the difference between the differentiated types of cells receives, as yet, no clear answer from the published account of these cultures.

The duality, or in some cases plurality, of cell-types in epithelia is such a universal phenomenon that it possibly has some deeper meaning than the mere convenience of the differentiation of the particular organ or tissue showing it. The accompanying Table I shows the cells which are present in some of the more conspicuous epithelia in the body, and there are certainly very few epithelia in which at least two types of cell cannot be readily demonstrated. It can, of course, always be argued that epithelia seldom have only one task to perform and therefore this histological complexity is solely determined by functional needs. On the other hand, in its evolutionary processes, Nature tends to build upon what is already present and she does so largely by continual processes of modification; it would thus be quite reasonable to suppose that, for some reason or other, epithelial cells in the vertebrates are not all fundamentally alike, but fall into two or more functional and

TABLE 4.I

Epithelium	*Cells*
Skin	Keratinizing, dendritic
Sweat glands	'Dark', 'light'
Mammary gland	Acinar and duct
Salivary glands	Mucous, serous, duct
Stomach	Surface mucous, neck mucous, peptic, oxyntic, argentaffin, argyrophil
Intestine	Goblet, brush-border, Paneth, argentaffin, mucous cells of Brunner's glands
Pancreas	α, β, exocrine, duct
Liver	Duct, hepatic (note also Kupffer cells)
Trachea and bronchi	Ciliated, goblet, basal, non-ciliated
Choroid plexus	Ciliated, polypoid
Kidney	Capsule, neck tubule, proximal tubule (segments 1 and 2), 'thin' loop of Henle, 'thick' loop of Henle, distal tubule, collecting tubule
Fallopian tube	Ciliated, non-ciliated
Vas efferens	Ciliated, non-ciliated
Uterus	Ciliated, non-ciliated, mucoid
Retina	Rod, cone
Olfactory organ	Hair, supporting
Gustatory organ	Hair, supporting
Auditory organ	Hair, supporting
Anterior pituitary	Basophil, acidophil, chromophobe
Thyroid	Chief, P.A.S.-positive
Parathyroid	Chief, eosinophil

perhaps structural groups, typified, for example, by serous salivary cells and mucous salivary cells or goblet cells and brush-border cells. It will be seen later that this duality or plurality is something which may arise as a consequence of some important and basic embryological processes which probably have, themselves, an earlier phylogenetic and originally functional origin.

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CHAPTER 5

THE GROWTH OF AMOEBOCYTES

IN cultures of many kinds of tissue, in addition to any growth of mechanocytes or of epitheliocytes which may occur, there often appear cells of yet another type. These cells are usually distinguishable in living cultures by the rounded form of their pseudopodia and by their irregular and membranous processes (Fig. 5.1); but they are most easily recognized by their movement when seen in cine-films of the growing cells. They are usually very actively amoeboid, sending out pseudopodia in any direction, so that they move in an apparently random manner, though in a general direction away from the central explant. Their more rapid movement usually causes the formation of a halo of these cells round the margin of any culture which contains them (see Fig. 2.1). Sometimes they remain motionless in a rounded condition for quite long periods. In active movement they are thus clearly distinguishable from the more static and more polarized or orientated mechanocytes on the one hand, and from the membrane-forming epitheliocytes on the other. It is a notable feature of these cells, and one which probably depends in part upon their manner of movement, that they mostly remain isolated from each other and normally show no tendency to stick together. They avoid each other's society as it were, so that in a culture in which these cells are numerous the cells are more or less evenly distributed over the surface upon which they are growing, and, it need hardly be said, that like the other cell types, they require a "wettable" surface before they display their amoeboid activity. Unlike the pseudopodia of mechanocytes, those found in this class of cell are less often pointed, but are more usually either rounded or lobose; they may often extend around a large part of the circumference of the cell, or even surround the whole cell in the form of an undulating membrane. On the other hand, under some conditions, the cells may lengthen out along one axis and assume a somewhat mechanocyte-like form; in this state they may easily be mistaken for mechanocytes though the rounded form of the ends of the pseudopodia generally remains and affords a means of distinction, when it can be seen (Fig. 5.2). Very small and fine filamentous pseudopodia are occasionally seen in active movement. It is obvious therefore that cells of this type are extremely variable in their morphology and very dependent on the properties of the medium in which they are placed (Parker, 1938).

Cells of this class are appropriately called amoebocytes and possess, in addition to those already outlined, other recognizable characters. Amoebocytes are likely to turn up in cultures of almost any tissue, and

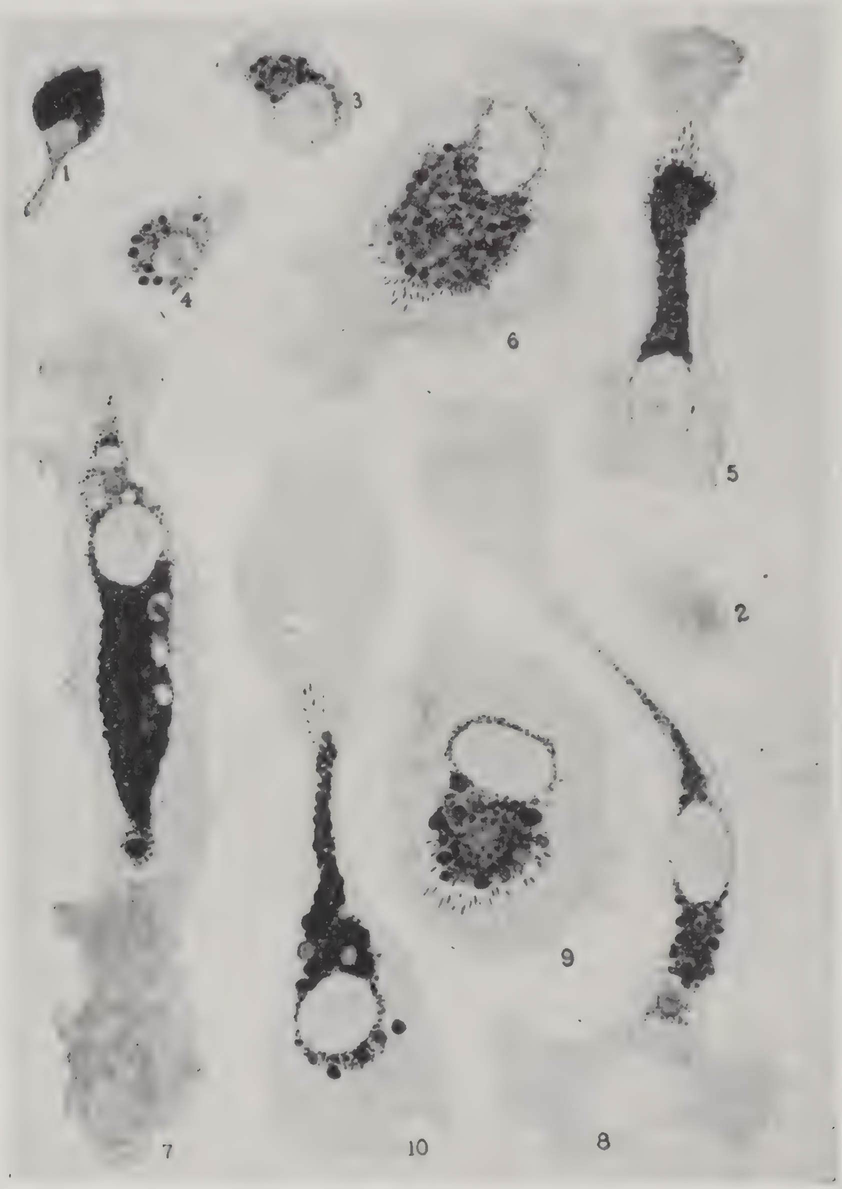


FIG. 5.1

Amoebocytes in tissue culture in plasma and embryo extract.
1. Blood monocyte. 2. Erythrocyte. 3–8. Blood monocytes in cultures of increasing age up to four days. 9–10. Tissue macrophages, after 24 hrs. in culture. (Carrel and Ebeling, 1926.)

this is not altogether surprising in that many of them are, in all probability, directly derived from the macrophages, which, of course, are almost universally distributed in the tissues of the bodies of the vertebrates. Like the macrophages they are in some conditions highly phagocytic and they can segregate such colloidal vital-dyes as trypan-blue, lithium carmine, and erythrolitmin. In tissue culture, they often have a great affinity for the basic dye, neutral red, which they can

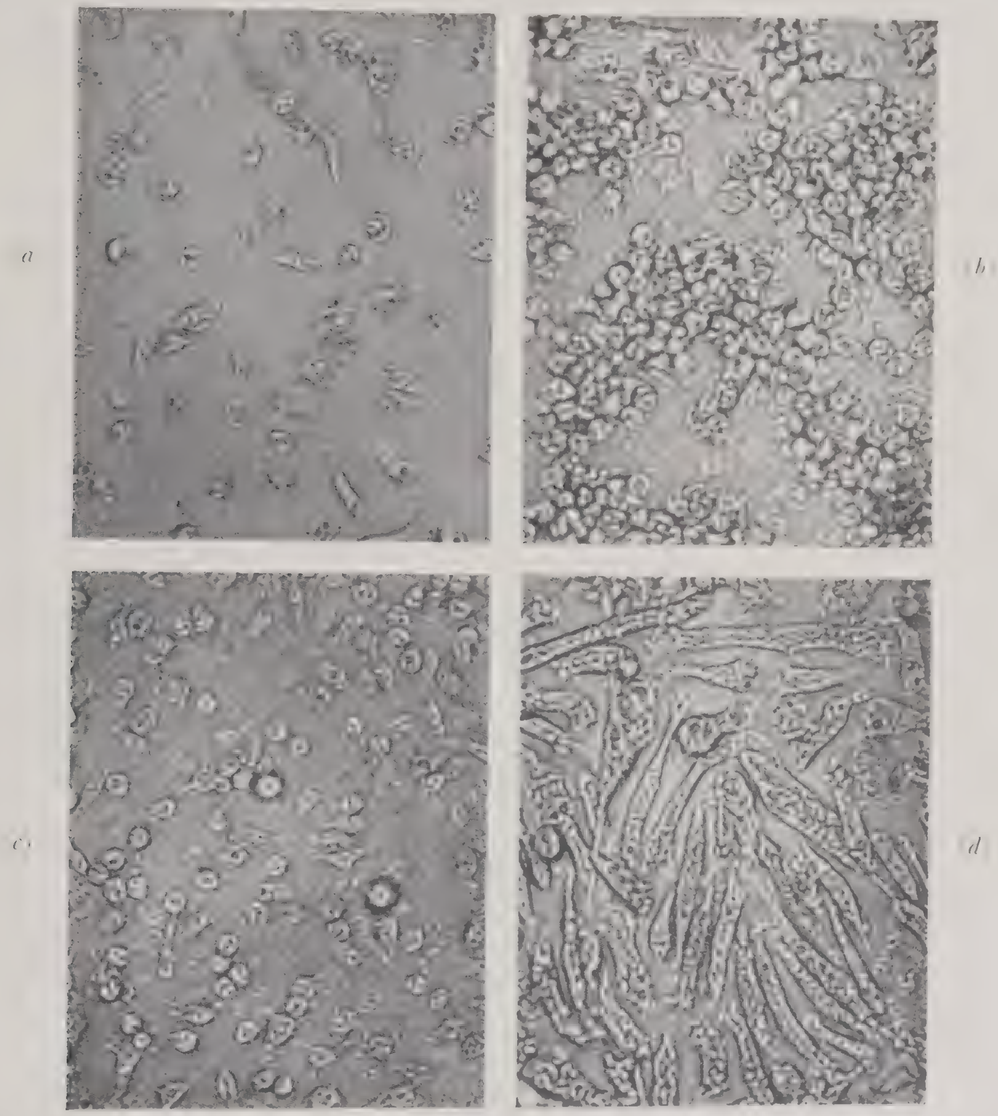


FIG. 5.2

Various forms assumed by chick monocytes when growing under different conditions. (*a*) in 20% serum. (*b*) in 80% serum. (*c*) in 50% serum. (*d*) in 50% serum + 2·5 mg/100 cc thyroxin. (After Carrel, see Parker, 1938.)

concentrate into their cytoplasm in a very few moments to a surprising degree (Carrel and Ebeling, 1926). This can be used as a ready guide to their presence in mixed cultures, as for example those obtained from the skin.

Numerous amoebocytes emerge from cultures of spleen and lymph nodes. In cultures of lymph from the thoracic duct the monocytes are clearly distinguishable from the lymphocytes and it is usually only the monocytes which survive after a few days in culture and by then they have become indistinguishable from amoebocytes derived from other sources. Amoebocytes also emerge from tissue cultures of the retina (Pomerat and Littlejohn, 1956) and brain, in which tissue they are normally present as the "microglia".

A very convenient source of these cells for cultures is the "buffy coat" obtained by centrifuging blood. If the blood plasma is carefully removed from the surface of the sedimented blood corpuscles and a drop of tissue extract dropped on to their surface, the buffy coat is then rapidly enclosed in a clot and can be removed as a whole, cut into fragments, and these fragments can then be cultured in plasma, just like the little pieces normally explanted from other tissues, either in flasks or hanging drops. Cells immediately wander out in all directions; granulocytes (i.e. neutrophil, eosinophil and basophil leucocytes), lymphocytes and monocytes all emerge actively and many erythrocytes are carried passively into the medium. After a few days, however, the granulocytes, erythrocytes and in most cases the lymphocytes are necrotic or already dead and disintegrated. The monocytes, however, persist and actively multiply. Most of them adhere to the surface of the glass or to that of the plasma and the former remain adherent to the glass after the plasma has been removed. In this position they may afterwards be kept in a fluid medium consisting of diluted serum alone, and in this, unlike fibroblasts, they continue to multiply so that their growth and general activity can be readily studied (Baker, 1933). Exactly similar cells can be obtained more directly from blood by albumen flotation, i.e. by suspending the cells in an albumen solution of the correct specific gravity and then centrifuging at the appropriate speed. If this is done from fowl blood, the monocytes are at first somewhat mixed with other blood cells and with thrombocytes but they are easily separated from these since the blood cells and thrombocytes do not stick to the surface of the glass as firmly as do the monocytes (Weiss and Fawcett, 1953) (Fig. 5.3).

These monocytes from the blood behave in exactly the same way as the macrophages described above as emerging from cultures of all sorts of other tissues, for these also survive well on glass in a fluid medium. In fact, under tissue-culture conditions there are no apparent differences between monocytes and macrophages (Jacoby, 1938), for the former readily develop the phagocytic properties of the latter. Furthermore, tissue culture shows up an amusing paradox concerning the classical terminology of the tissue macrophages. These are often – and somewhat arbitrarily—divided into active or free macrophages and fixed macrophages, the latter name referring to those phagocytic cells which appear in tissues in a more or less extended form as though they were part of an established order in the tissue. The former refers to the roundish cells which may be seen loose, or unattached to the tissue framework, as though they were moving about or being carried in the tissue fluid or lymph. In fact, the "fixed" macrophage may often be really in a state of active amoeboid movement, phagocytosing particles as it goes, and it may be anything but "fixed"; whereas the round "active" macrophage may simply be indulging in one of the more or less prolonged periods of rest which are typical of these cells at least in culture conditions, where periods of active migration alternate with periods of stagnation, often with the cell in a rounded-up condition. This is but one of the many instances where the opportunity to see the cells alive has largely altered the interpretation to be placed upon a given fixed, and thus falsely static, histological situation. The only other comparable conditions for observing cells alive are those found in such places as the tadpole's tail, in the artificial culture chambers in rabbits' ears (Sandison, 1928; Clark, Clark and Rex, 1936; Ebert, Sanders and Florey, 1940) or in the dorsal skin of mice (Algire, 1943). In such preparations, macrophages can again be clearly seen if trypan-blue is used; and, interestingly enough, under these conditions, even the extended macrophages are much more static than they are in cultures.

(*a*) FIG. 5.3 (*b*)

(*a*) Culture of monocytes and thrombocytes from chick blood. (*b*) "Pure culture" of monocytes now adhering to a glass surface. (Weiss and Fawcett, 1953.)

The fact that these cells not only live but multiply freely in a medium of serum alone, without any period of acclimatization, distinguishes them at once from the mechanocytes which only multiply extremely slowly under such conditions, unless they are specially "trained to it". Most macrophages or amoebocytes continue to multiply for several days in serum, and when they have ceased to divide, renewal of the serum is sufficient to start up growth and division once more. The lag period after feeding and before the first cell divisions appear is longer than the lag period when mechanocytes are treated with embryo juice and is generally about 20 hours, or rather less if the serum is frequently renewed (Jacoby, 1941) (Fig. 5·4). There are, however, some races of amoebocytes, e.g. those obtainable from the peritoneum of the guinea-pig, which are somewhat different in morphology and apparently have no power of multiplication *in vitro* (Jacoby, 1957) (Fig. 5.5). Treatment of amoebocytes with embryo juice produces fatty and rather round cells and many of them degenerate; only a very limited growth occurs in its presence. By contrast, mechanocytes may become slightly more fatty than normal in embryo juice, but they certainly thrive in its presence, and under ordinary conditions need it, or some tissue extract like it, for their multiplication.

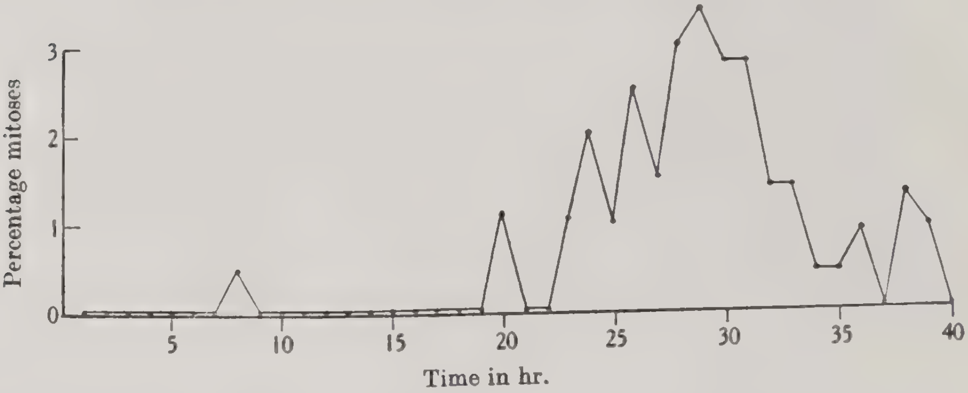


FIG. 5·4

Effect of feeding with 25% serum on the growth of tissue macrophages *in vitro*. The serum was added at zero time and mitoses start again at 20 hours. (Jacoby, 1938.)

The property which amoebocytes possess of separating from each other in a given colony has further repercussions, for when several colonies are planted in the same dish, they show, unlike those of mechanocytes, no tendency to fuse, and in fact there often remains a cell-free zone between the colonies (Carrel and Ebeling, 1922) (Fig. 5.6). This is made even more remarkable by the fact that the average velocity of movement of amoebocytes is higher than that of mechanocytes and they quickly extend all over a vacant surface. Not only that, but colonies of amoebocytes can grow actively within the substance of a plasma medium and invade it to almost any depth. Mechanocyte colonies grow with less and less vigour, the deeper they are planted in

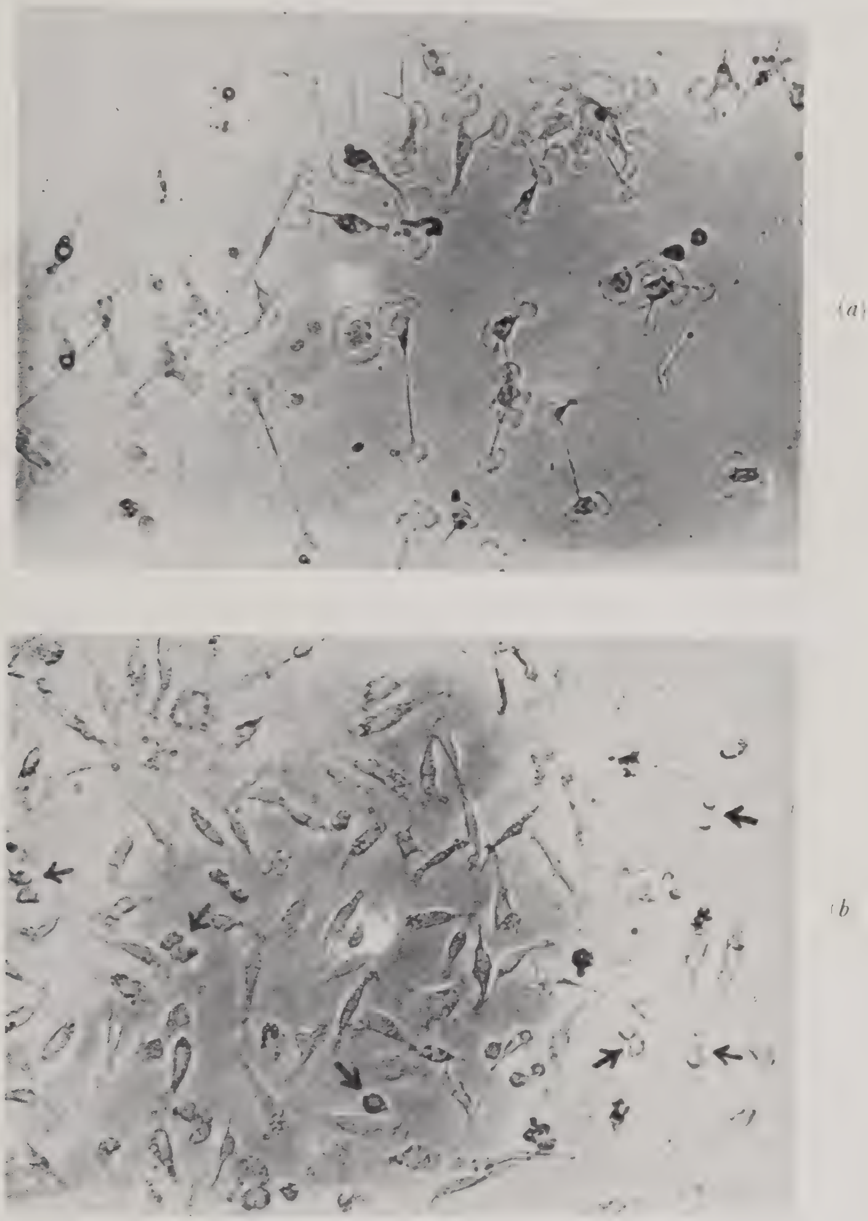


Fig. 5.5

Amoebocytes from the peritoneal cavity of the guinea-pig (*a*) compared with those from the hen (*b*). (Jacoby, 1957.)

a tube of coagulated medium, and they are highly dependent on an adequate access to oxygen; amoebocytes, on the other hand, extend widely into the medium more or less independently of their depth in the coagulum (Fischer, 1928) (Fig. 5·7).

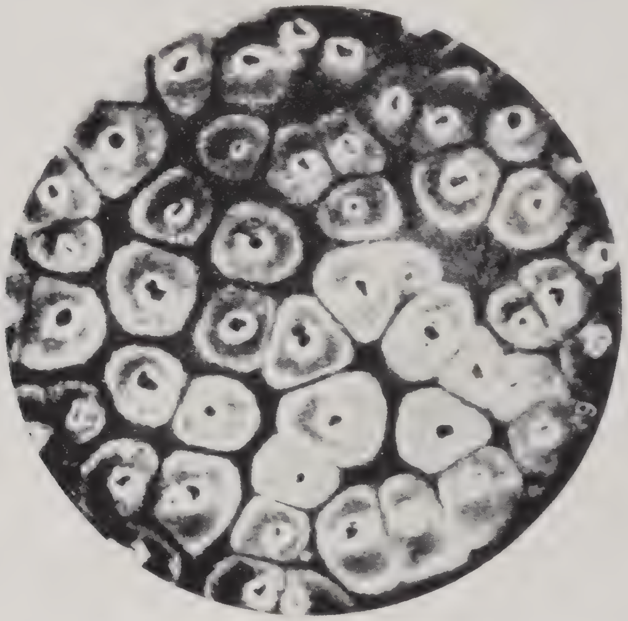


Fig. 5.6
Colonies of monocytes maintaining zones of separation between the colonies. (Carrel and Ebeling, 1922.)

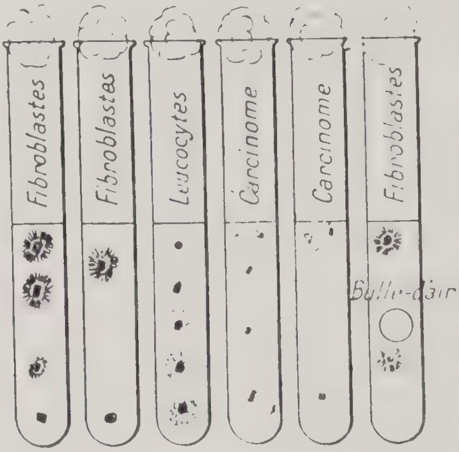


Fig. 5·7
Diagram showing the manner in which different types of cells grow at different depths in a plasma coagulum. (Fischer, 1928.)

In colonies planted in the substance of a clot, it quite often happens that tubular cavities are formed while the plasma is coagulating. These cavities are the seat of an interesting piece of behaviour on the part of the amoebocytes, for these cells may flatten on the surface of such cavities and line them with cells of "epithelioid" form (Hueper and Russell, 1932) (Fig. 5.8), rather in the same way as some mechanocytes can behave to form endothelia. Generally, however, the cells are not so closely adherent unless they are very much crowded together.

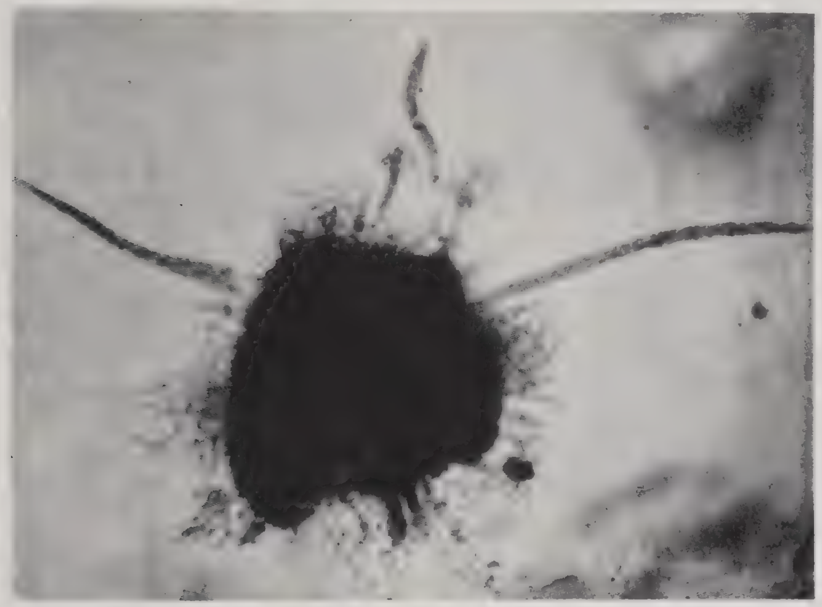
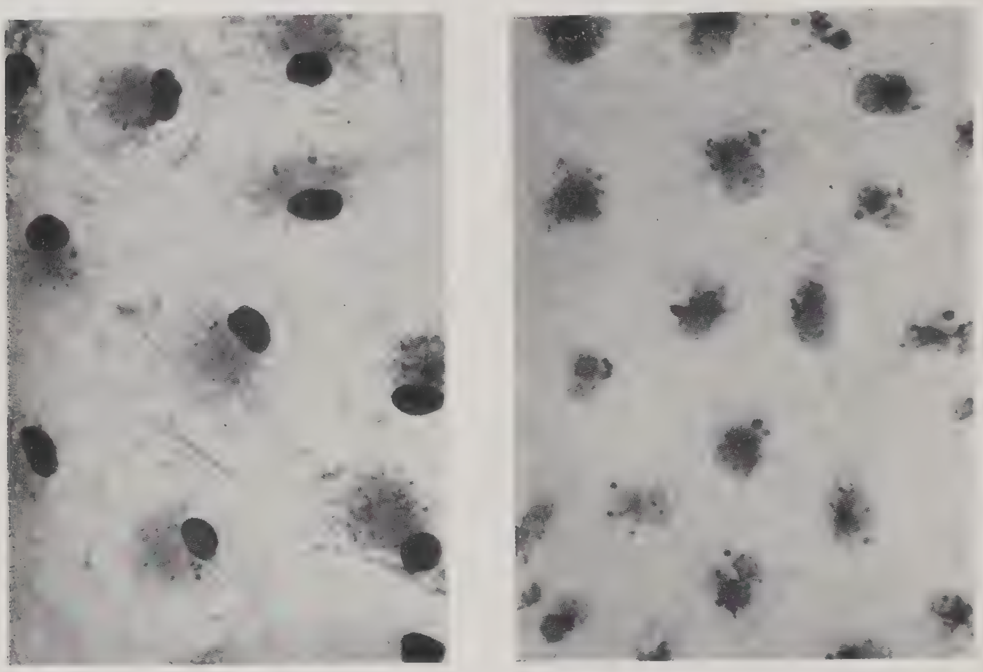


FIG. 5.8
A culture of leucocytes with seven capillary-like formations lined with cells extending into the plasma clot. (Hueper and Russell, 1932.)

Since amoebocytes are, in general, capable of segregating vital dyes, this behaviour in lining tubules is perhaps suggestive of the manner of formation of those endothelia in the body which are exceptional in the rather loose and mesh-like distribution of their cells and in their capacity to segregate lithium carmine and the like, namely the endothelia of the bone-marrow sinusoids, of the liver sinusoids (the Kupffer cells) and of vessels in the adrenal cortex. When amoebocytes adopt the epithelioid form (Fig. 5.9a) they acquire a very conspicuous centrosphere and this is strongly positive for acid phosphatase (Fig. 5.9b) and also reacts positively to the periodic Schiff reaction, even after digestion with saliva (Weiss and Fawcett, 1953). This, incidentally, is an interesting example of cells increasing the amount of an enzyme during culture *in vitro*, for the monocytes freshly drawn from the blood do not give any reaction for acid phosphatase. Perhaps the enzyme is correlated with the increased phagocytic activity in the tissue culture conditions.


(*a*) FIG. 5.9 (*b*)

(*a*) Amoebocytes in the epithelioid form, each with a conspicuous centrosphere. (*b*) Acid phosphatase in the region of the centrosphere in "epithelioid" monocytes. (Weiss and Fawcett, 1953.)

Amoebocytes in the "epithelioid" form may often have several nuclei (Fig. 5.10), and the formation of giant cells with many nuclei by progressive fusion of individual cells occurs readily *in vitro* (Lewis, 1927

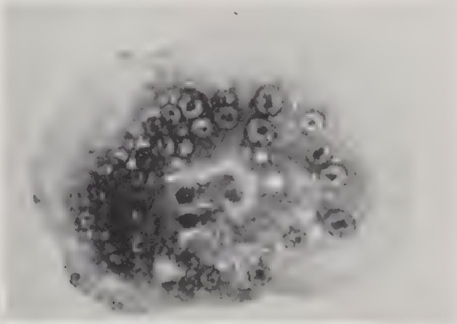


FIG. 5.10
Giant amoebocyte with many nuclei. (Weiss and Fawcett, 1953.)

particularly in response to foreign bodies (e.g. tubercle bacilli, lycopodium powder) and to low oxygen tension (Barta, 1925) and possibly low pH (Fig. 5.11). It is curious that these cells, which normally behave as though they repelled each other, when they actually make contact in this way should fuse their cytoplasm, particularly since as epithelioid sheets they seem able to make some sort of contact with their neighbours without fusing with them. When fragments of bone are explanted in plasma there wander out from them into the medium, within the first few hours, not only osteoblast cells, of mechanocyte form, but also cells which rapidly get larger by fusion with other cells of the same type and which may eventually have as many as an hundred nuclei (Hancox, 1946) (Fig. 5.12). These cells, which sometimes emerge in the multinucleate form, presumably correspond to the osteoclasts of the bone. Cinephotography shows that they move with the characteristic gait of amoebocytes rather than with that of

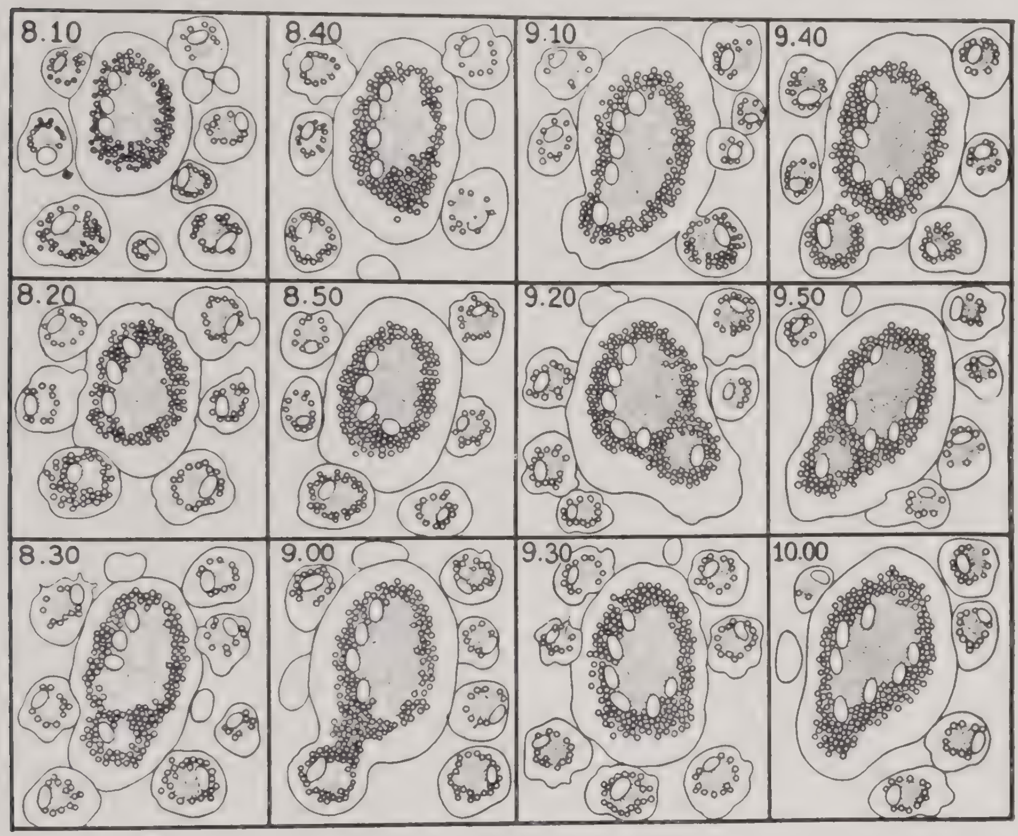
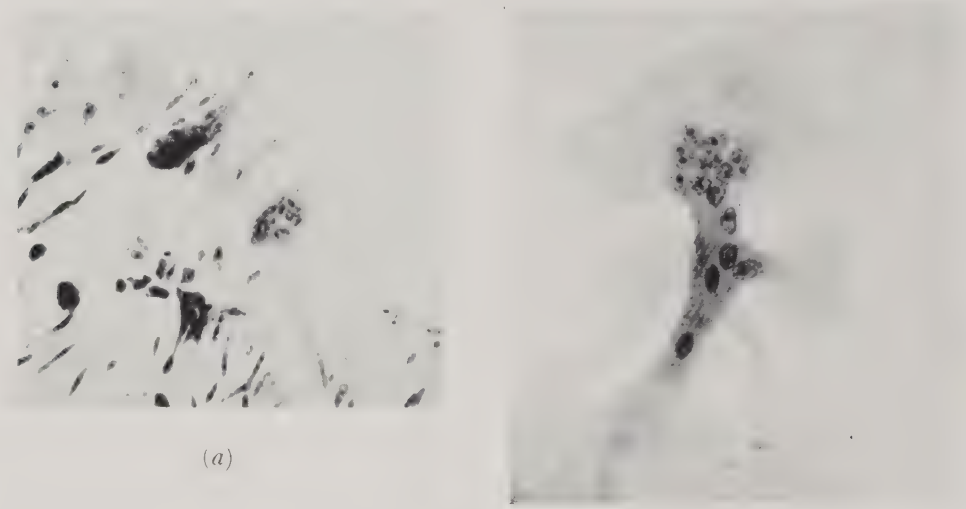


FIG. 5. 11

Semidiagrammatic drawings of amoebocytes fusing to form a giant cell. Numbers represent times. (Lewis, 1927.)

mechanocytes, the margins of the cells often showing well-developed undulating membranes (Hancox, 1949).

It is of course well known that osteoclasts appear when bone is being broken down *in vivo,* though the proof that they are the causative agents is lacking. Nevertheless, experiments *in vivo* and *in vitro* with vitamin A may be illuminating. It has already been noticed that high concentrations of vitamin A in the medium of bone and cartilage cultures cause the disappearance of the bone matrix without the appearance of numerous osteoclasts (Fell and Mellanby, 1952). On the other hand, restoration of vitamin A to the diet of A-deficient puppies leads to a sudden thinning of the bones in certain regions of the skeleton where it had previously not been eaten away as it would have been in a normal puppy. In these regions numerous osteoclasts immediately appear (Mellanby, 1947). In the light of the experiments on the metaplasia of epithelia with high doses of vitamin A in the medium (Fell and Mellanby, 1953) and of the suggestion that mucus-secreting cells absorb sulphur as sulphate (Jennings and Florey, 1956; Kent *et al.* 1956), it seems possible that amoebocytes may perhaps do something similar and that the sudden accumulation of osteoclasts when bones are being dissolved away or remodelled in the body as a whole, where there are, of course, numerous circulating monocytes, etc., may be connected with the sulphur-containing breakdown products of the bone or cartilage matrix. In cine-films of tissue cultures of bone grown with pieces of parathyroid tissue the matrix of bone



(*a*) (*b*)

FIG. 5.12

Osteoclasts and osteoblasts emerging from fragments of bone in tissue culture (*a*). The former readily form giant cells (*b*). (Hancox, 1946.)

disappears, and numerous osteoclasts can be seen in the regions of bone removal (Gaillard, 1955). Striking as this picture is, it does not in fact differentiate between cause and effect, and has to be interpreted in terms of the other experiments in which cartilage-resorption, and in some cases bone-resorption, occur in response to high doses of vitamin A in tissue cultures of long bones, and when few osteoclasts or giant cells appear.

Pure colonies of amoebocytes have not been observed to produce any fibres or "ground-substance" into the medium. However, when cultures are made from the buffy coat of the blood it sometimes happens that some colonies after a time begin to produce mechanocyte-like cells and a tissue with collagen fibres (Maximow, 1928; Hulliger, 1956; Paul, 1958). The capillary-like processes sometimes produced in cultures of blood also occasionally have a fibrous backing (Parker, 1934). This has often been taken as evidence for the transformation of amoebocytes into mechanocytes, but since the same thing does not occur among the amoebocytes cultured from lymph (Hulliger, 1956) the presence of some unrecognized mechanocytic cells in the blood (endothelial cells?) is to be suspected (Paul, 1958), though the possibility of a transformation certainly cannot be excluded.

It is said that colonies of leucocytes planted near colonies of mechanocytes encourage the growth of the latter by the production of food substances or "trephones" (Carrel, 1922), which are advantageous to the fibroblasts, but it is not really clear which cells among the "leucocytes" are responsible for this effect nor whether it is more than the effect of the autolysis of the less viable leucocytes, since extracts of tissue which have been allowed to autolyse produce notable stimulation of the growth of mechanocytes. The occurrence of numerous macrophages in a repairing wound after the first invasion of the neutrophil leucocytes suggests a possible stimulating action of these cells on the main tissue-forming cells or fibroblasts, but until the nature and origin of trephones is more firmly established some caution is necessary in the matter. Meanwhile amoebocytes, unlike both epitheliocytes and mechanocytes, have not been shown to form any staple product, comparable with keratin and collagen. The origin of fibrinogen and serum proteins is still problematical: that they arise in the liver is probable, but whether the hepatic cells or the Kupffer cells are responsible still remains uncertain.

Again unlike mechanocytes, amoebocytes are able to start colonies moderately easily from isolated cells and these can be well maintained in fluid media. Such media may be entirely synthetic and in that case amoebocytes will survive in them for some months, but unless there is at least 10% serum in the medium the cells tend to go slowly down-hill and survival beyond a few months is impossible (Jacoby and Darke, 1948). With some serum present, however, growth is apparently unlimited. Serum is also known to increase the phagocytic capacity of macrophages.

It has been noticed in colonies of mechanocytes and particularly in those from muscle, that cells, which have all or at any rate many of the characteristics of macrophages, may appear quite suddenly in the outer growth-zone, and this has led to the belief that mechanocytes can become macrophages (Fig. 2.1). This transformation may or may not be the correct explanation for this phenomenon, and the whole problem will be discussed in some detail later, but it should be emphasized that "pure" colonies of mechanocytes are extremely difficult to obtain with certainty; nests of macrophages, or even individual macrophages, might well remain enclosed in the tissue for long periods and, under conditions favouring the growth of mechanocytes, they would themselves not be much encouraged to proliferate. However, if the embryo juice for some reason became diluted or less potent, then the macrophages might well flourish and migrate out. It has been found that the numbers of amoebocytes appearing in muscle cultures can be increased by the addition of choline and of certain quaternary ammonium compounds (e.g. trimethyl-ethyl-ammonium chloride) to the medium (Thomas, 1936; Chèvremont and Chèvremont-Comhaire, 1945). The interpretation of this phenomenon may not be the simple one, namely that mechanocytes turn into amoebocytes. Indeed, the application of certain quaternary ammonium compounds to fibroblasts during their culture, *in vitro*, while cinematograph records of them were being made, was not observed to bring about any morphological changes in the growing cells or any change in their movement except those concerned with toxicity at the higher dose levels (Pomerat and Willmer, unpublished observations).

A physiological feature frequently and easily seen in the amoebocyte, though not exclusive to it, is the ability of the cell to engulf some of the surrounding fluid and to enclose it within the cytoplasm as a vacuole (Lewis, 1931). After a time this vacuole slowly decreases in size and becomes more highly refractile (Gey, Bang and Gey, 1954; Rose, 1955); eventually it disappears. The process is known as pinocytosis and seems to occur especially when there are lamelliform membranes or "ruffles" round the main body of the cytoplasm. It is not known what significance is to be attached to the process, but it is suggestive of a feeding method, if the imbibed water is then excreted. Electron-microscope pictures of macrophages often show invaginations of the cell surface that give the impression of penetrating deeply into the cell in such a way that their ends may become nipped off as vacuoles (Palade, 1955). These invaginations may well be concerned with pinocytosis.

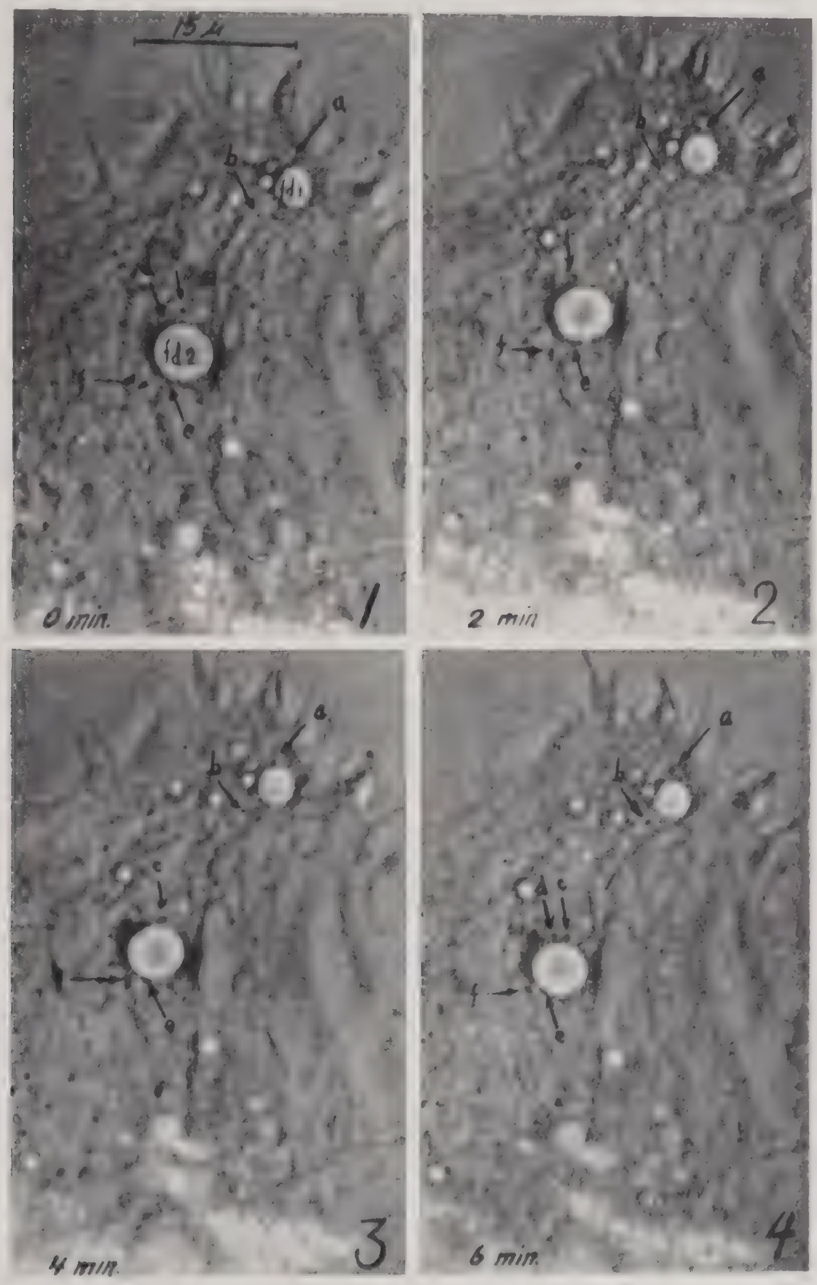


Fig. 5.13

Pinocytosis, with microkinetospheres (*a f*), in "Hela" cells. Photographs at 2 min. intervals. Phase contrast. fd1, fd2, Pinocytosis vacuoles. (Rose, 1957.)

It is possible that this process is something which is more easily observed in tissue cultures, since the cells are then often excessively flattened and the normal traffic of fluid in and out of the cell may thus be adversely affected.

In some cells in tissue culture, e.g. Hela cells, (which were actually derived from epithelial cells of a carcinoma of the cervix of the uterus) it has been observed (Rose, 1957) that pinocytosis vacuoles are often "met" in the cell periphery by refractile granules (microkinetospheres) originating near the nucleus and passing outwards to the periphery (Fig. 5.13). These granules become associated with a vacuole and then accompany it back to the centre of the cell where the vacuole itself may be greatly reduced in size and disappear as such. While this phenomenon was first observed in the rather abnormal Hela cells, it has since been seen to occur in normal macrophages.

Amoebocytes, therefore, appear by derivation, behaviour, growth-potency and many other characteristics, to constitute a third clear-cut family of cells and in general they can be readily distinguished from both mechanocytes and epitheliocytes.

It may be difficult immediately to classify any one particular cell on purely visible characters alone, as definitely belonging to this family or to that, but when all the characteristics of the cell's behaviour, including its relationship with other cells, are noted, then the cell can be classified with a greater degree of certainty. The great majority of cells, as they are seen in the early stages of unorganized growth in cultures from a wide variety of tissues, would then be found to belong to one of the three main categories which have been discussed so far.

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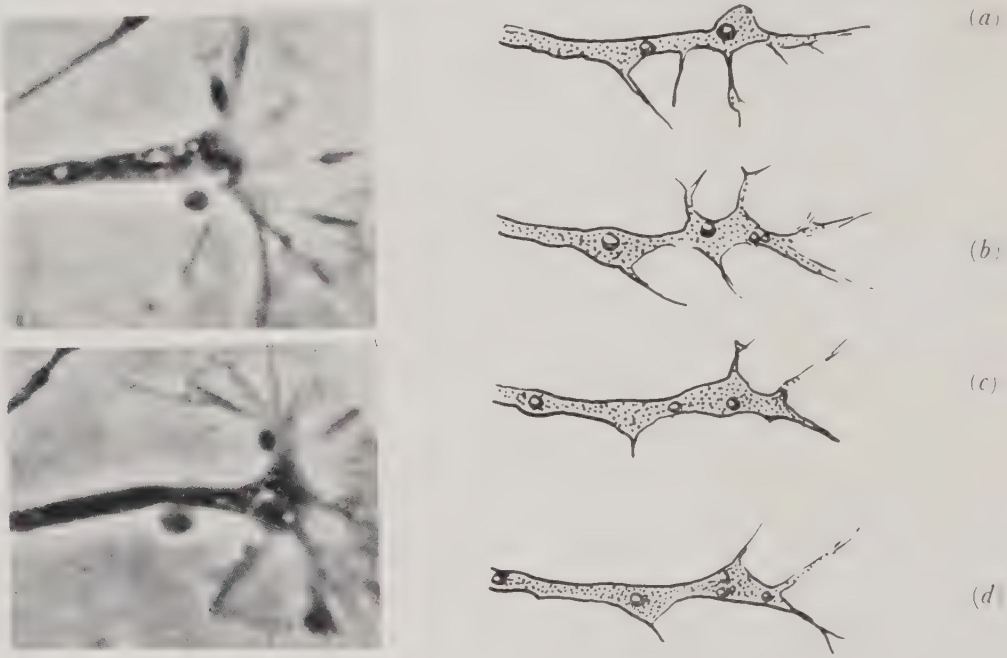
Chapter 6

NERVE CELLS, NEUROGLIA AND SCHWANN CELLS

Although the technique of tissue culture was developed in order to investigate the behaviour of nerve cells, and it immediately solved some fundamental problems concerning the neurone, it has not, since those early days, been so extensively used for the investigation of the behaviour of nerve cells as it has been for that of mechanocytes and of the other types of cells already discussed. Perhaps one of the main reasons for this lies in the fact that "unorganized" growth, in the sense of the proliferation by mitosis, of nerve cells, hardly occurs in tissue culture. It is said that nerve cells while still in the neuroblast stage, grow as an epithelial sheet, but as soon as their differentiation has started, then this no longer happens. On the other hand, in old cultures of brain there do sometimes emerge sheets of epithelial cells, the origin of which has not been definitely established. It is possible that they may represent the undifferentiated ependymal cells (Olivo, 1927).

Nerve cells *in vitro* often send out long and branching processes, each ending in amoeboid pseudopodia. Speeded-up cine-films show very active movement in these outgrowing processes which, in some cases, is extremely similar to the movement of the pseudopodia of amoebocytes even to the extent of the rapid enclosure of droplets of fluid by pinocytosis (Hughes, 1953) (Fig. 6.1.). These droplets of fluid are passed quickly up the extended nerve process towards the cell body and if the nerve fibre is cut off from its parent cell, the absorption of fluid and the passage of the vacuoles up the fibre may continue for some time, before the whole disconnected terminal portion degenerates in the expected manner. During this time numerous vacuoles collect at the cut, so that it is quite clear that, at any rate under these conditions of outgrowth *in vitro*, these nerve-cell processes are constantly collecting fluid from their surroundings and passing it up towards the cell body. Since it has also been shown that when nerves are regenerating, after section *in vivo*, they tend to force fluid or cytoplasm outwards down the nerve fibre towards the cut, so that a constriction acts like a dam (Weiss, 1944a) and so that the end of the nerve fibre proximal to the cut may enlarge into a distinct bulge (Young, 1945; Weiss and Hiscoe, 1948), it is difficult to reconcile these two apparently diametrically opposed observations. Either there must be movement simultaneously in both directions along a nerve fibre, and, after all, there is nothing inherently unlikely about this since it is obviously occurring in the less elongated pseudopodia of other types of cells, or else it may be that such factors as the age of the tissue, external conditions, rate of growth and even the type of nerve cell under observation are the determining factors. There is as yet no information as to whether dendrites and axons behave alike or differently in this way in tissue cultures. In fact, it is not always easy to decide which is which, though there are some nerve cells which in tissue culture show one process, generally posteriorly situated, which is different in kind from the other and anterior processes (Fig. 6.2). The anterior processes, as the cell advances into the medium, are presumably the dendrites in these cells, and it is probably these which show pinocytosis. It would be interesting to know how the axon behaves in this respect.



(a) (b)

Fig. 6.1

"Pinocytosis" by the processes of outgrowing neurites. (*a*) Membranous neurites with accumulating droplets. (*b*) Drawings of a neurite showing the positions of the vacuoles at different times. Interval between a and b 48 sec., between b and c 12 sec., and between c and d 12 sec. (After Hughes, 1953.)

During prolonged culture under conditions which favour survival, both in hanging drops of cerebrospinal fluid (Martinovic, 1932) and in flasks and roller tubes with heparin plasma or serum and with low concentrations of embryo juice (Costero and Pomerat, 1951), nerve tissue survives and differentiates to a remarkable degree (Figs. 6.3, 6.4). The cells may slowly move out into the medium under these conditions, and in some cases the central explant may eventually thin out sufficiently to enable the living cells to be studied in some detail. Cell division does not usually occur in such cultures, although curiously enough, it has been described in cells emigrating from explants of adult human sympathetic ganglia and which are apparently nerve cells (Murray and Stout, 1947) (Fig. 6.5). Some of these cells also become multinucleate giant cells (Fig. 6.6).

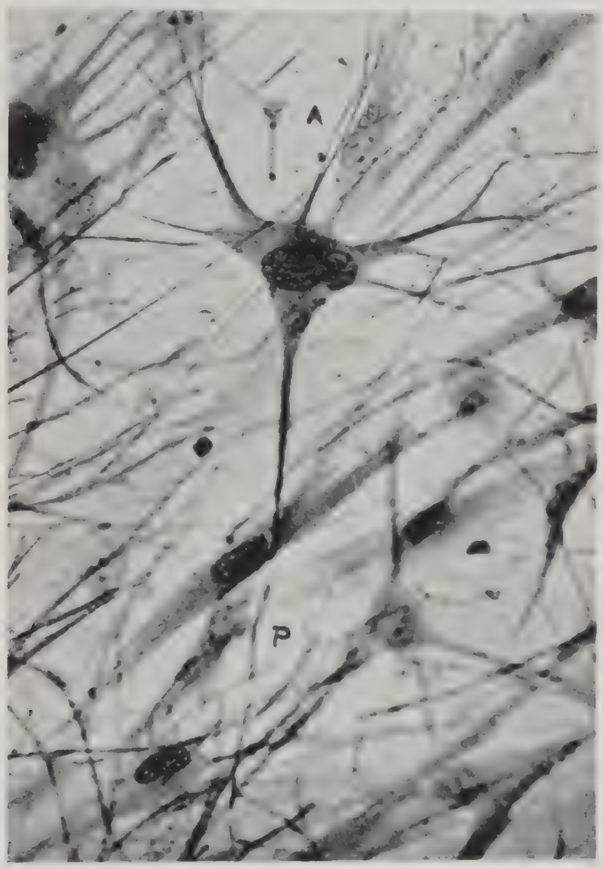


Fig. 6.2
Sympathetic ganglion cell in tissue culture showing distal or advancing processes (A) and a trailing proximal process (P). (Murray and Stout, 1947.)

This case emphasizes the importance of examining different parts of the nervous system, perhaps rather more carefully than has been done in the past, with respect to the sort of behaviour which can be expected from the different histological types of cells. Sympathetic ganglion cells, derived embryologically from the neural crest, may have somewhat different properties from those cells which are developed from the neural tube proper. Their very migration during embryonic development suggests greater powers of movement than those possessed by many nerve-cell bodies. It certainly seems to be true that the sympathetic ganglion cells also migrate outwards in tissue cultures and that many of them assume the form of rather large essentially fan-shaped cells with a definite polarity (see Fig. 6.2). In this way, their general form appears to be very like that of mechanocytes. The cell has a series of sharp and pointed distal (or anterior) processes and it may or may not drag a long proximal (posterior) process behind it. This sort of behaviour has also been observed in Purkinje cells from the cerebellum and in some cells which emerge from cultures of the cerebral cortex (Hogue, 1947; Martinovic, 1932). The distal processes are presumably, again, the dendrites and the proximal process, which is single and somewhat different in appearance, is the axon. The cells from the sympathetic ganglia, which incidentally have, like those from other neural tissues, a strong affinity for methylene blue, and also

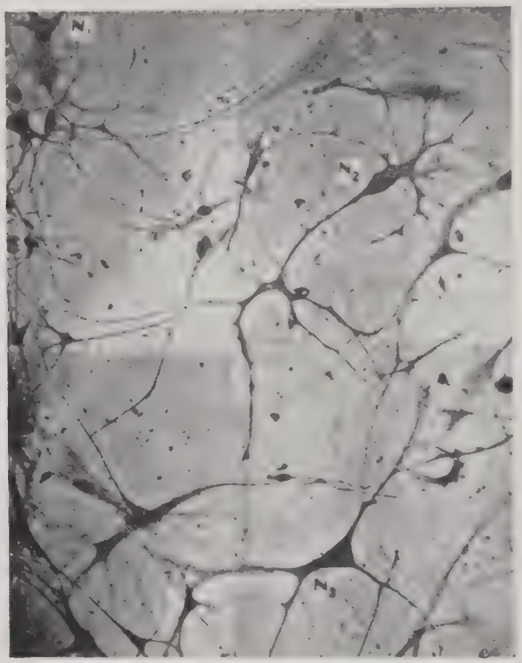


Fig. 6.3

Nerve cells differentiating in the outgrowth from prolonged culture of the cerebellum. Three nerve cells (N_1, N_2 and N_3), can be seen with their processes. Silver preparation. (Costero and Pomerat, 1951.)

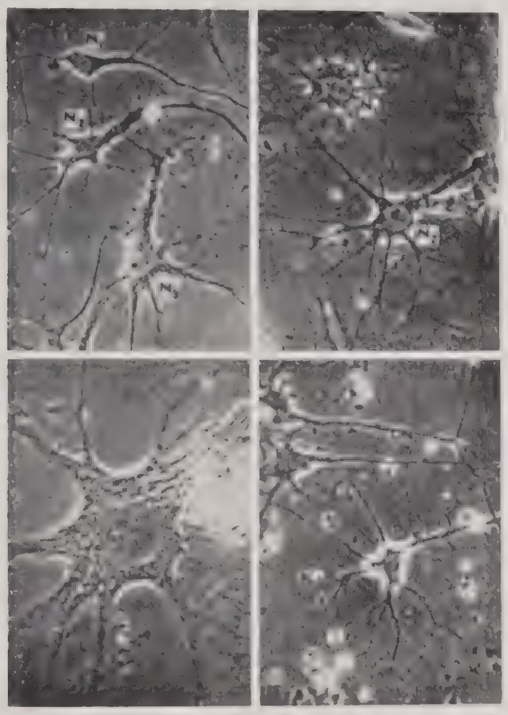


Fig. 6.4
Phase-contrast pictures of living nerve cells in prolonged cultures of cerebellum. (Costero and Pomerat, 1951.)

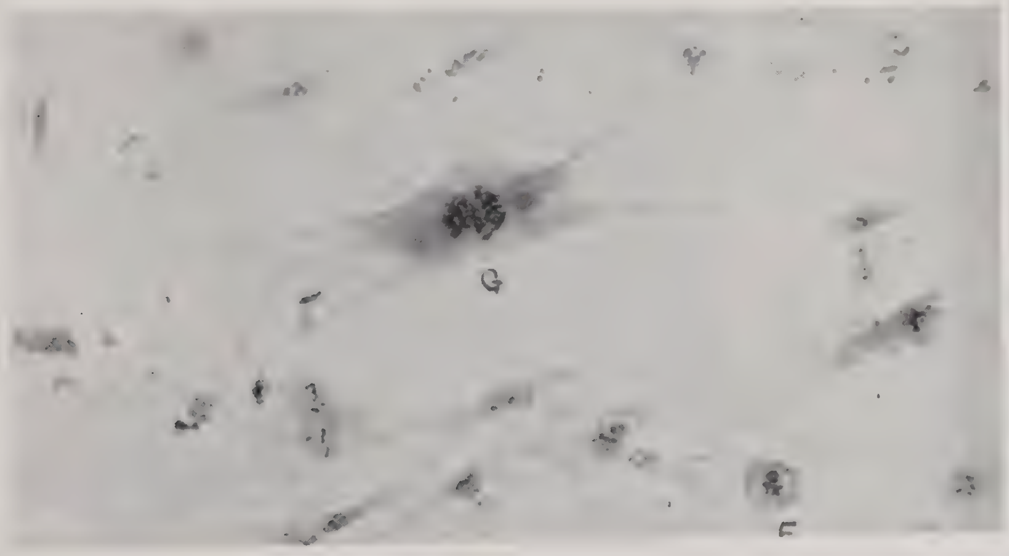


Fig. 6.5
Dividing neurone in culture of adult human sympathetic ganglion. Note the polyploidy. (Murray and Stout, 1947.)

for silver stains when fixed (Fig. 6.7) have, as mentioned above, been shown upon one or two occasions to withdraw their processes to a greater or less extent and then to divide in tissue culture. After division, as with mechanocytes and other cells, their rate of migration is increased. In some of these cells there is a definite increase in the amount of chromosome material as compared with that in the accompanying fibroblasts (see Fig. 6.5).

No definite Nissl's substance can be detected in nerve cells during the first phases of their life in tissue culture, and ultraviolet light shows a rather uniformly high absorption by the whole cytoplasm (Koenig and Feldman, 1953). Later, however, the typical arrangement of Nissl's substance may develop (Deitch and Murray, 1956) (Fig. 6.8). Neurofibrillae are probably structures which are produced by an intensification of the orientation of long molecules or micelles in the cytoplasm, during the process of fixation or other treatment, and are thus probably similar, in this respect, to the fibres of the mitotic spindle which can be made to appear and disappear within the living cell. Some orientated material is usually visible in spinal ganglion cells (Weiss and Wang, 1936), and it may be significant that these cells do not readily migrate from the central explant under tissue culture conditions. The mitochondria or similar granules in nerve cells often show a concentric arrangement around the nucleus (Murnaghan, 1941) which could easily help to give rise on fixation to the neurofibrillar pattern of the cytoplasm which characterizes many nerve cell-bodies (Levi and Meyer, 1937). In other systems, e.g. sea-urchin embryos, (Gustafson and Lenicque, 1952) the development of mitochondria often precedes the elongation or stretching of protoplasm as though some phase of mitochondrial activity favoured the production of the fibrous or orientated proteins. A curious feature of many nerve cells, and also of some epithelial cells, is that the nucleus may often be seen, with the help of time-lapse cine-photography to be rotating within the cytoplasm (Murnaghan, 1941).

As mentioned above, some nerve cells and fibres growing in tissue culture have, as elsewhere, a special affinity for methylene blue, and this allows them to be distinguished fairly readily from the other types of cells which may also grow out from nerve tissues, both *in vivo* and particularly *in vitro*. Neutral red is also copiously picked up by some cells both *in vivo*, e.g. the large ganglion cells in the retina, and *in vitro*, e.g. the spinal ganglion cells (Murnaghan, 1941), and it would be interesting to see if this property could be correlated with other features in the behaviour of nerve cells. Not all nerve cells do it.

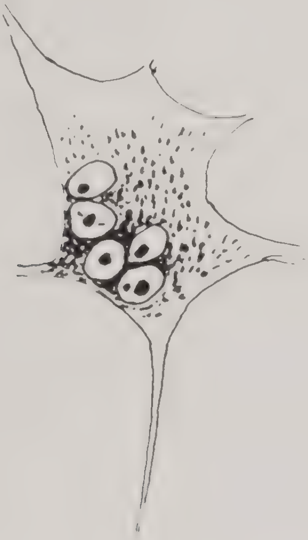


Fig. 6.6

Multinucleate giant cell from neurone growing from human sympathetic ganglion in tissue culture. (After Murray and Stout, 1947.)

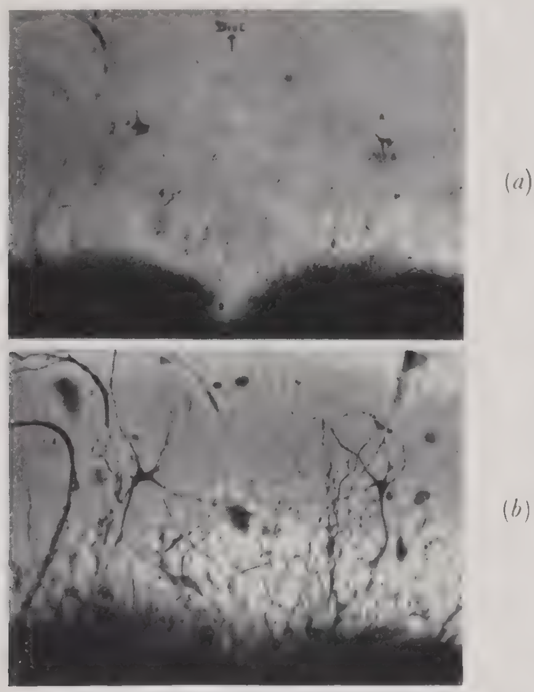


Fig. 6.7

Sympathetic ganglion cells in the outgrowth of a tissue culture before (*a*) and after (*b*) fixation and silver impregnation. (Murray and Stout, 1947.)

After staining with methylene blue, nerve fibres often show, both *in vivo* and particularly *in vitro*, very marked globular swellings at intervals along their length. The significance of these is not yet known; they may be enlarged and made more conspicuous by the treatment with methylene blue but they are certainly detectable also in the untreated nerve fibres. It is possible that they may be connected with the passage of fluid droplets along the fibre. Many of the fibres which grow out in culture are undoubtedly naked cell processes—dendrites and axons, but quite often neuroglia cells or Schwann cells may accompany the true nerves, creep along them and sometimes invest them in much the same way as they have been observed to do in the tail of the living tadpole (Speidel, 1933). Myelin formation has been observed in tissue cultures on several occasions though it usually only occurs after prolonged cultivation (Peterson and Murray, 1955).

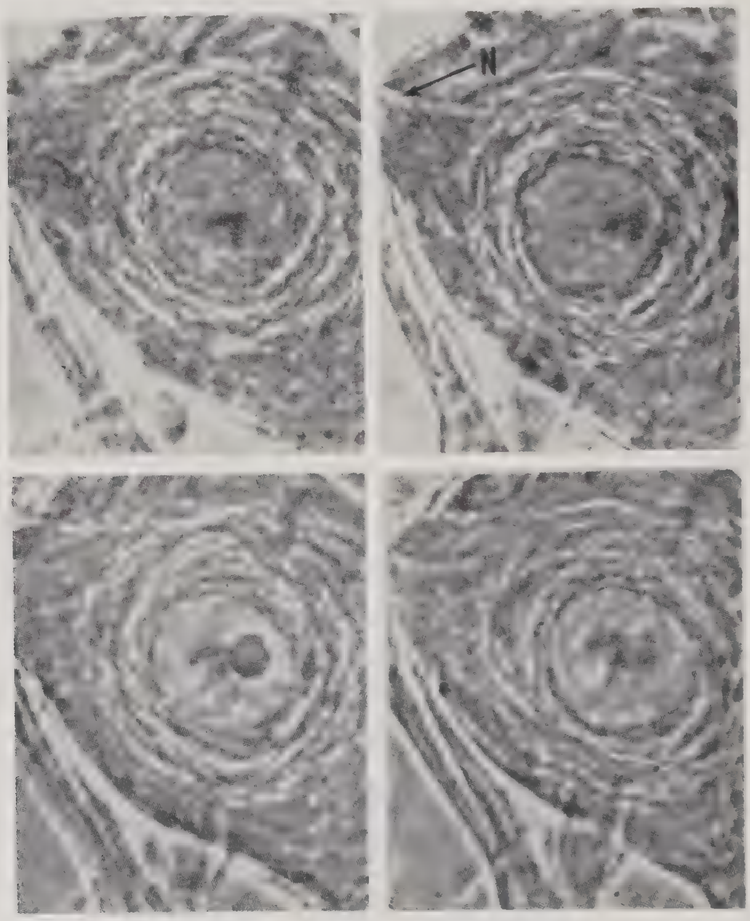


Fig. 6.8

Nissl's substance in spinal ganglion cell in tissue culture. (Deitch and Murray, 1956.)

While the property of conduction is the essential and distinguishing feature of nerve cells and one which is very difficult to investigate by tissue-culture methods, there is no reason to believe that it is the exclusive property of one type of cell only; *a priori*, not all nerve cells should necessarily be regarded as being alike except in the fact that they can transmit excitation or inhibition, i.e. in the fact that they conduct. Many of them conduct "impulses", but it is by no means certain that they all do so. Tissue-culture studies have already emphasized some of the differences between different groups of nerve cells but there has so far been very little serious attempt at any comprehensive classification according to their general physiological properties. For example, in the retina, only the large ganglion cells pick up methylene blue (Francis, 1953) and in fixed preparations certain silver methods also pick out only the large ganglion cells while leaving the small ones unstained. A similar silver method stains the nerve cells in parasympathetic ganglia but not in sympathetic ganglia (Nonidez, 1939); so there are clearly differences between the cells in these groups but as yet the nature of these differences is not known. In the amphibia the Mauthner cells degenerate under the action of thyroxin while other cells in the neural tube are stimulated to increased mitotic activity (Weiss and Rosetti, 1951). There are indeed many morphological types of nerve cells but it is possible that they may be found to fall into a more limited number of main groups when investigated more thoroughly from the metabolic and physiological angle, e.g. adrenergic, cholinergic, fast or slow conducting, showing "all or none" or graded activity, etc.

Schwann Cells and Neuroglia

The Schwann cells have been studied *in vitro* by making tissue cultures of portions of peripheral nerve trunks after these have previously been severed from all connection with their nerve-cell bodies, and after sufficient time has been allowed for degeneration of the nerve fibres to begin. This degeneration brings the Schwann cells into a state of migratory activity and under these conditions they wander out from the cut ends and grow in the tissue-culture medium generally in the form of a network. They do not readily emerge from normal nerve fibres (Abercrombie and Johnson, 1942). Cultures have also been successfully made from Schwann-cell tumours and it seems to be fairly clear from these cultures that Schwann cells may behave in one of two ways (Murray, Stout and Bradley, 1940) (Fig. 6.9). Perhaps there are, indeed, two types of Schwann cells. Those which are associated with amyelinate fibres are characterized by a distinct bipolarity and the possession of very long thin processes: the nucleus is generally broader than the average width of the cell, so that its presence and position is marked by a bulge. Branches from these cells tend to sweep gracefully away from the cell body and form contacts with neighbouring cells of similar type or they may creep along nerve fibres (Fig. 6.9 *a*).

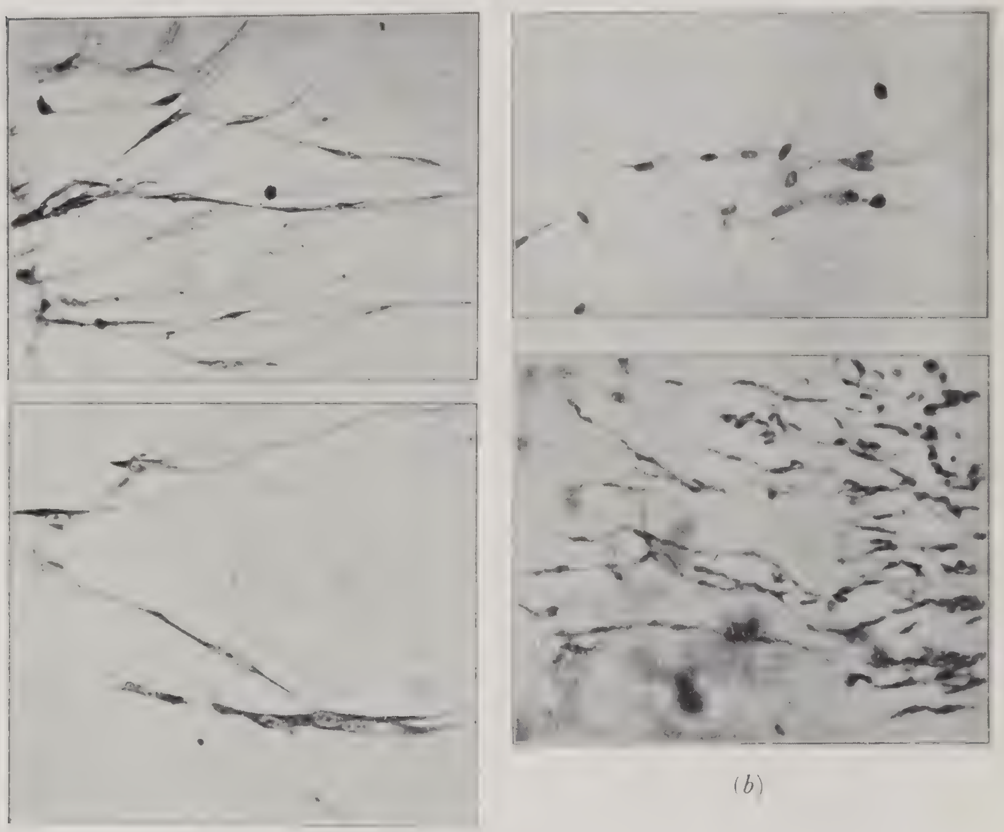

(*a*) (*b*)

FIG. 6.9

Figures illustrating the difference in behaviour of Schwann cells in culture. (*a*) Mechanocyte-like growth. (*b*) Amoebocyte-like growth. (Murray, Stout and Bradley, 1940).

The Schwann cells which are associated with myelinate fibres are generally more branched cells and their processes, which are not so slender and fine, tend to be expanded into membranes (Fig. 6.9 *b*). This is particularly noticeable in the cells which migrate out from the spinal ganglia and dorsal roots in a medium of serum and Tyrode (Weiss, 1944b) (Fig. 6.10). These cells closely resemble amoebocytes in appearance (see Fig. 5.5), and the serum medium which was used is one which would favour the growth of such cells. Unlike amoebocytes they form a network with processes from other similar cells, but the whole net appears to be rather irregular. The significance to be attached to these observations is by no means certain. It may simply be that there are two forms of behaviour possible for the Schwann cells, either of which may be set up by external factors. Alternatively, it may be much more fundamental and indicate two separate races of cells both of which are usually classed under the generic term of Schwann cells. It has been known for many years (Langley and Anderson, 1904) that when nerves are cut and then sutured together again, the preganglionic fibres in the autonomic nervous system will not grow down postganglionic paths, nor will postganglionic fibres regenerate down preganglionic or somatic fibres except to supply blood vessels. Most efferent somatic fibres behave like preganglionic fibres, but afferent somatic fibres do not make functional connections with efferent somatic fibres. All these observations indicate differences in character between the Schwann tubes in the various nerves. In the autonomic system the relationship can probably be summarized by the statement that cholinergic fibres do not regenerate down the tubes of adrenergic fibres nor *vice versa*.

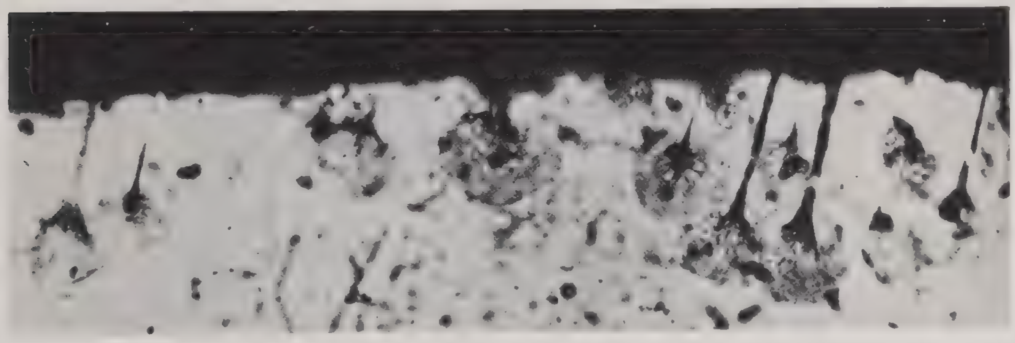


Fig. 6.10
Amoebocyte-like cells emerging from spinal nerve in culture. (Weiss, 1944b.)

In comparing the illustrations of Schwann cells, shown in Fig. 6.9, one is reminded of the patterns of growth of mechanocytes on the one hand and of amoebocytes on the other. A notable difference, however, in the second case is in the fact that the Schwann cells normally form a network, while the amoebocytes tend to remain discrete. Schwann cells are clearly different both from fibroblasts and from macrophages as such, but it seems possible that the two forms of their behaviour may be in some ways similar to the sort of patterns of behaviour seen respectively in mechanocytes and amoebocytes. Weiss and Wang (1945) have, indeed, described a series of changes of form from Schwann cells to fibroblasts and to macrophages in tissue cultures.

Among the other "supporting" cells of the nervous system, mention may first be made of the astrocytes (Fig. 6.11). These appear, in long-continued tissue cultures, to be cells normally characterized by very large undulating membranes; these membranes are of peculiarly unstable character, and they very easily break down into a series of radial processes with more or less amoeboid ends (Costero and Pomerat, 1951). The changes may perhaps lead to the production of "protoplasmic" and "fibrous" astrocytes as they appear in histologically prepared sections of the central nervous system. A similar state of affairs on a more modest scale is seen in the modification of the lamelliform membranes of amoebocytes. However, before any serious attempts are made in the interpretation of the nature of neural and

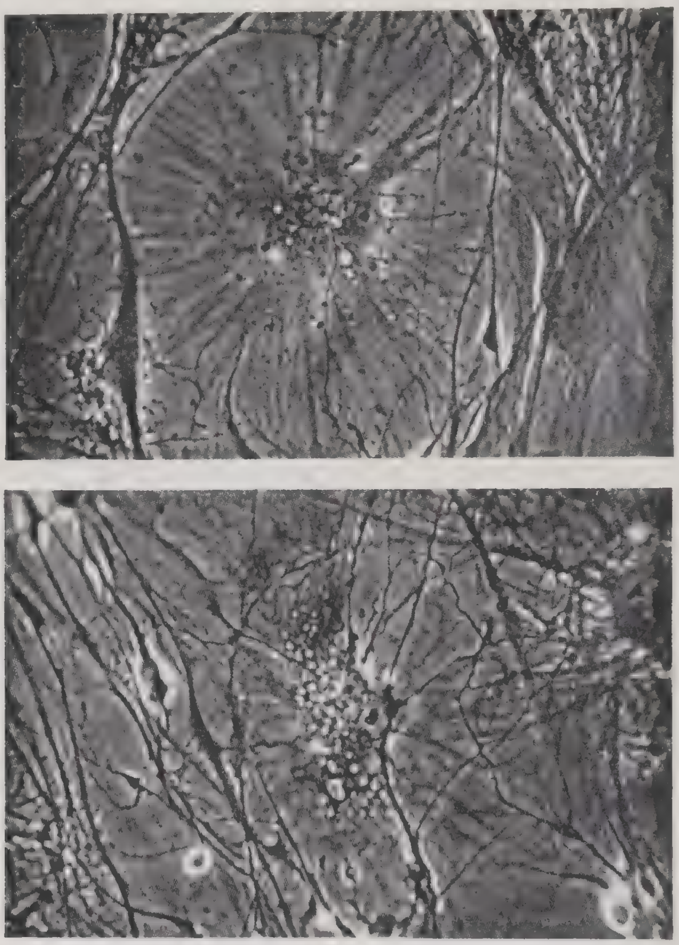


Fig. 6.11

Living astrocytes in tissue culture. Damage leads to dissection of the membranes, as in the lower figure. (Pomerat, 1952.)

neuroglial tissue from the appearance of the cells alone, attention should perhaps be called by way of caution to the extraordinary branching cells (illustrated in Fig. 2.11) which appeared in old cultures of fibroblasts (Parker, 1933); morphology without physiology can be very deceptive.

The oligodendroglia are readily recognizable in tissue cultures (Fig. 6.12). They emerge mainly as bipolar cells or sparsely branching cells with small refractile cell bodies and they often collect into groups (Hogue, 1950). They have also been frequently observed to lie with their cell bodies adjacent to those of nerve cells. In fact, it has been suggested on these grounds that there is some kind of symbiosis between the cells in these two classes. Once again the speeded cine-film has brought to light an unexpected form of behaviour, for oligodendroglia cells are slowly and rhythmically contractile, with a period of about five minutes (Lumsden and Pomerat, 1951). Recent studies with the electron microscope (Luse, 1956 a and b) suggest that oligodendrocytes are partly responsible for the myelination of the axons in the central nervous system; their processes, in this respect, behave in a very similar manner to those of the Schwann cells; that is to say they wrap themselves around or fold themselves repeatedly against the axon and so form a series of more or less concentric layers (Geren, 1954). Astrocytes appear to have a similar function, and this again raises the question of why there are two sorts of cell for this function.

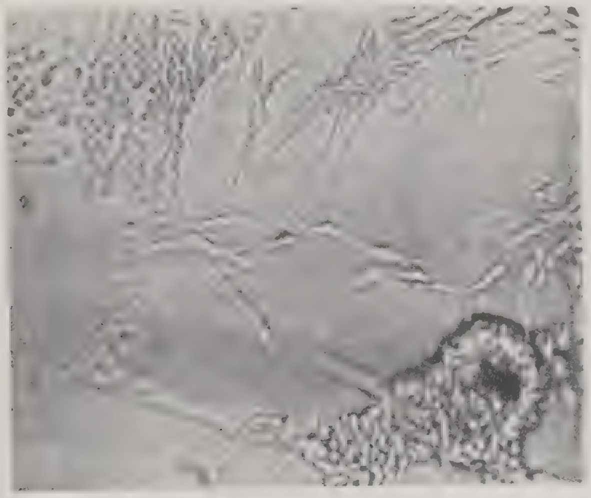


FIG. 6.12

Nerve cell and numerous oligodendroglia cells in tissue culture of cerebellum. (Hogue, 1947.)

The multipolarity of astrocytes and the bipolarity of oligodendroglia again suggest comparisons with amoebocytes and mechanocytes respectively. So far as the available figures for the relative frequency of astrocytes and oligodendrocytes (Glees, 1955) and for those for the extent of acetylcholine synthesis in different parts of the central nervous system (Feldberg and Vogt, 1948) can be compared, there does not appear to be any immediately obvious correlation between them. It is not possible, therefore, to correlate on these grounds either type of neuroglia cell specifically with a particular type of nerve fibre or of chemical transmitter. Perhaps such a correlation should not be expected since nerve cells themselves may contribute to the distribution of these substances (transmitters and their associated enzymes), and probably in an inverse relationship with the neuroglia cells. However this may be, the neuroglia cells and Schwann cells certainly have common points of interest; they each show two forms of behaviour and are each concerned with myelination and there is some sort of "symbiosis" between them and the nerve cells.

The microglia have already been mentioned in the discussion on macrophages, with which they seem to be identical or, at any rate, very closely related.

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CHAPTER 7

THE BASIC CELL TYPES

THE behaviour of the different types of mammalian and avian cells and tissues in culture has been described in some detail in order to give a general picture of cell behaviour and to emphasize the outstanding physiological qualities of the cells. All that has been said so far is thus in the nature of an introduction to an approach to cell physiology which has been much neglected in the past, but which now can be tackled with more hope of advantage than ever before. Classical cytology (meaning by this cell physiology rather than cytological genetics) necessarily had to base most of its conclusions and its hypotheses on the knowledge gained by the study of fixed and stained materials or by histochemical methods on sections or teased preparations of tissues. It was thus essentially cell morphology. Tissue culture, however, not only accentuates the vital qualities of cells but it allows them to be studied under conditions over which the experimenter has rather more control, though this amount of control is by no means as complete as would be desirable. Tissue culture has the advantage that processes can often be studied while they are actually in progress, and not by the interpretation of a series of stages alone. Cells can be seen in action and, what is more important, direct tests can be made upon them without involving all manner of secondary effects which may emanate from the responses of other tissues in the body to the experimental or to other, irrelevant, conditions.

The results of tissue culture work which have received most comment in this account are those which have been obtained by the techniques encouraging "unorganized" growth, since these allow the individual cells to be more easily and directly studied. Under these conditions, it must again be emphasized, the cells are not usually functioning exactly as they do *in situ*, and sometimes they are indeed behaving very differently. There is certainly modification, and probably some simplification of their normal behaviour. All the data gained by tissue-culture methods have therefore to be sifted and weighed against data from other sources before there is any hope of developing what may be regarded as a balanced view of cell behaviour. In many instances, of course, the results of tissue-culture experiments involving "organized growth" have been able to make extremely valuable contributions, and now that somewhat simpler methods are available for these experiments, organized growth is likely to be more studied in the future and to provide more and more relevant information about cell behaviour. Certainly the cells are likely to be functioning more normally under these conditions, but at the same time the degree of isolation of the cells is much less and the problems and effects of cellular interaction, on the one hand, and the necessity for finally fixing and sectioning the tissues for examination on the other, both put severe limitations on the method for studies on the physiology of the individual cell. Nevertheless, data obtained by "organ culture" methods must also be woven into the tapestry of knowledge obtained by other methods, and for some purposes such data are likely to be the most reliable.

Before proceeding to discuss the import of the tissue-culture data in a more constructive manner, it will be advisable to review some of the main concepts that have emerged so far.

The salient fact for the purpose of this discussion is that when tissues from the various organs and structures of the human body, and indeed of all the vertebrates which have been investigated, are grown *in vitro*, under conditions which favour unorganized growth, then the emigrating cells simplify their form and their behaviour so that they approach one or other of a very limited number of patterns. If it were only the outward form of the cell which tended to conform to a type, then, in the light of the great variability which is noted in tissue cultures under experimental conditions, this tendency to adopt one or other of a few main types, though interesting in itself, would not perhaps have much general significance. However, when the characteristic forms of cells in tissue culture are also accompanied by specific biochemical differences, by peculiar methods of locomotion, by distinct patterns of cell contacts and by somewhat parallel types of behaviour under pathological conditions in the body, it then becomes obvious that the meaning of these "tissue-culture" groups lies deeper and is a matter worthy of investigation since it may throw new light on fundamental cell properties and on some essential processes in cell differentiation.

The Nature of the Differentiation

The characters and properties of the cell groups have been collected together and summarized in Table 7.1; this table deals only with the three main groups: the mechanocytes, epitheliocytes and amoebocytes. The question of the cells constituting the neural tissues will be taken up again later. It is fairly clear from the table that there are sufficient

TABLE 7.I

Epitheliocytes	*Mechanocytes*	*Amoebocytes*
Grow as a sheet with intimate cell contact.	Grow as a network exhibiting contact inhibition. Isolated cells survive with difficulty.	Grow as isolated cells.
Pseudopodia, at edge of growth only, mostly lamelliform but sometimes pointed.	Characteristically pointed pseudopodia.	Characteristically lamelliform and rounded pseudopodia.
Cells attached to coverslip locally only—tendency for attachments to break.	Cells in culture attached to coverslip at extremities only. Tough cell membrane. (Chambers & Fell, 1931.)	Cells may be attached to surface more completely. Less rigid cell membrane. (Chambers & Fell, 1931.)
Colonies fuse.	Colonies fuse.	Colonies remain isolated. (Carrel & Ebeling, 1922.)
Gliding movement of the sheet, following extension by the peripheral cells.	Gliding movement of cells with polarity.	Amoeboid movement often without marked polarity.
Best growth on a surface.	Limited penetration into a plasma coagulum. (Fischer, 1930.)	Penetrate plasma coagulum to all depths. (Fischer, 1930.)
Some epithelial cells phagocytic *in vivo*. Not normally so *in vitro*.	Not normally phagocytic. Do not segregate vital dyes very actively.	Usually phagocytic. Active segregation of vital dyes.
Can pinocytose.	Can pinocytose.	Active pinocytosis.
Do not normally require embryo juice. (Liver inhibited by more than 20.%)	Require embryo juice or the like for cell multiplication. Embryo juice increases movement.	Become fatty and quiescent with embryo juice.
Grow in serum.	Cell multiplication not normally possible for long in serum. (Parker, 1938.)	Growth and multiplication of at least some, though not all, amoebocytes in serum. (Jacoby, 1937, 1957.)
Produce Carcinomata.	Produce Sarcomata.	Produce Leucaemia. (Round cell sarcomata ?)
	Cause differentiation of epithelial cells (e.g. Grobstein, 1953). Survive and differentiate in serum.	No evidence that they cause epithelial differentiation.
Mucosubstances may collect in the cytoplasm. In other cases, they may be extra-cellular.	Mucosubstances mostly produced externally to the cell.	Mucosubstances collect in the cytoplasm.
Tend to produce keratin.	Tend to produce collagen.	

TABLE 7.I—*continued*

Epitheliocytes	*Mechanocytes*	*Amoebocytes*
Uninhibited by hexenolactone. (Medawar *et al*, 1937, 1943.)	Inhibited by hexenolactone (Medawar *et al*, 1937, 1943.)	—
—	—	Encouraged by choline and quaternary NH_3. (Thomas, 1937.)
—	Inhibited by 1/3,000,000 As_2O_3. Killed by 1/800,000 As_2O_3. (Fischer, 1930.)	Unaffected by 1/800,000 As_2O_3. (Fischer, 1930.)
—	Do not support Rous chicken sarcoma virus. (Carrel, 1926.)	Support Rous chicken sarcoma virus. (Carrel, 1926.)
Some epithelia support fowl-pest virus (Hallauer, 1931.)	Do not support fowl-pest virus. (Hallauer, 1931.)	Do not support fowl-pest virus. (Hallauer, 1931.)

distinctive properties to justify this broad classification into three groups, though it is not really possible to say wherein the main differences lie, and there is no one feature which can be made absolutely diagnostic for purposes of classification. One may hazard a guess that the nature of the surface membrane is probably a primary factor in causing some of the changes. This same conclusion was reached by Loeb (1920) as the result of his extensive investigations into the behaviour of the amoebocytes from the blood of *Limulus*, the King Crab. These fascinating and very versatile cells have at times many of the characteristics of the amoebocytes of vertebrates but are probably rather more plastic. In support of the idea that the cell surface is important it is known that such a simple but "surface active" substance as sodium oleate in the medium of fibroblasts encourages their phagocytic powers and alters the type of movement of the cells, thus rendering them more like macrophages (Fischer, 1930). Similarly it is tempting to think that the liberation of mucoprotein by mechanocytes into the surrounding medium, as opposed to its storage within the cell by certain epitheliocytes, is perhaps related to stereo-chemical properties of the cell membranes. Incidentally, electron-microscope photographs of the boundaries between epithelial cells suggest that in some cases the actual cell membranes may be in contact, and often the membranes interdigitate in a most complex pattern, as in the tubule cells of the kidney. The so-called cement substance as described, for example, in the prickle cell layer of the skin, may well be contained within vacuoles which according to these figures, appear to lie within the cell (Pease, 1951) though other figures would suggest a different interpretation (Weiss, 1958). The binding together of cell to cell in many epithelia certainly depends on the action of calcium ions and perhaps of magnesium also. When Ca^{++} and Mg^{++} are removed from the medium the binding between the cells becomes weaker and the cells tend to fall apart, a process which can be assisted in some tissues by the presence of trypsin and elastase (Moscona and Moscona, 1952; Rinaldini, 1958). The actions of these substances are in all probability on the cell surface in the first instance.

Sudan Black and Baker's haematein test (combined with the use of suitable solvents) indicate the presence of phospholipins at the cell boundaries in the epidermis in the form of bead-like structures, but their significance is not yet established. Furthermore, the cause underlying the blackening of the boundaries of contact by silver nitrate is not yet established, and in many cells the blackening may be restricted to certain very limited regions of the cells' surface which persist when the contact is broken (Robinow, 1936).

All these observations point to the cell surface as a dominant feature of epitheliocyte behaviour. The contact inhibition described for fibroblasts (Abercrombie and Heaysman, 1952) and the mutual repulsion of amoebocytes together with their facility in pinocytosis, segregation and phagocytosis, stress the same point with regard to the other groups of "tissue-culture" cells.

It is unlikely, however, that the surface membrane is the only factor concerned in differentiating between these three types of cell, though it certainly accounts for certain aspects of their behaviour. The cell surface is apt to vary in relation to the surrounding medium, but Figs. 2.1 and 2.2 show that epitheliocytes, mechanocytes and amoebocytes may exist as such simultaneously in the same medium. It must be emphasized also that an epithelial cell in the body may often have different surface properties over different parts of its surface yet it remains an epithelial cell. These local differences may be determined in part by the immediate surroundings of the cell; the example has already been given of a secreting epithelial cell which has one surface exposed to the tissue fluid, several surfaces in contact with neighbouring cells, and another surface in contact with the lumen of the gland; all these surfaces appear to have different permeability relationships. In part, however, the difference may originate within the cell itself and be determined by, or related to, the "polarity" of the cell. Certain amoebae, for example, even in a uniform environment, may have lobose pseudopodia at one end and pointed filiform pseudopodia at the other. Epithelial cells from the developing amphibian larva when isolated into a saline medium, may show cilia at one end and pseudopodia at the other (Holtfreter, 1947b) (Fig. 7.1). The surface of the amoebocyte can be shown to be equally variable; filiform pseudopodia sometimes replacing undulating membranes, and both giving way to a more "limax" type of amoeboid movement with rounded "bleb" or drop-like pseudopodia. From these observations, and many more like them, it becomes obvious that within each of the three families of cells there is plenty of variability in the nature and behaviour of the cell surface both in space and in time, yet the three groups of cells maintain their differences in a general way; and even when growing under identical conditions, both colonies and individual cells usually maintain their own characteristic pattern of growth and behaviour. Each responds in its own way to changes in the medium. In other words, although behaviour may be much modified by the conditions of culture, the cells of the different types maintain certain intrinsic characteristics that speak of fundamental differences and of differences which are maintained. It may often be difficult at any one moment to distinguish between individual cells belonging to two different types, but it is not generally so when dealing with colonies of cells or when their behaviour can be observed over a period of time. The cells of one type have one set of intrinsic characters and those of another have another set, and these differences are maintained in spite of the fact that either cell may be modified by external events. From a single amoebocyte enormous numbers of similar amoebocytes can be cultured, and such cultures do not normally produce mechanocytes or epitheliocytes. The same is probably true of mechanocytes, but the establishment of pure colonies of these from single cells (i.e. clones) is a much more difficult and uncertain event. Many of the so-called pure strains of fibroblasts now in existence show cells, after

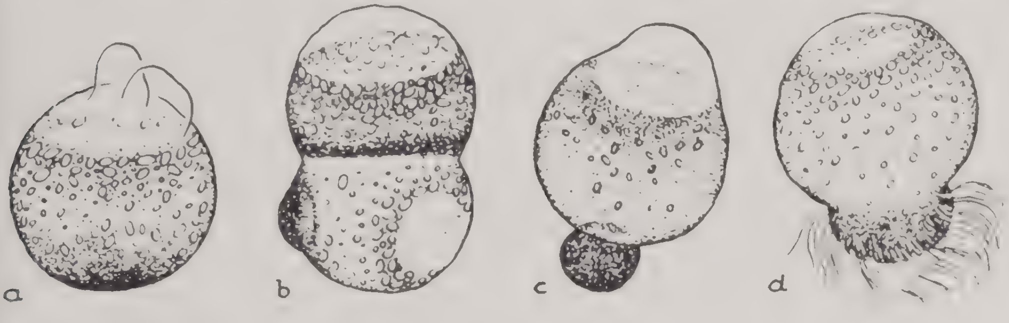

FIG. 7.1
Polar organization in isolated amphibian epidermal cells, the former distal pole being marked by pigment and occasionally (*d*) by cilia. (Holtfreter, 1947.)

prolonged culture, which it would be difficult to classify as either mechanocytes, amoebocytes or epitheliocytes (Figs. 7.2. 7.3). The meaning of this change is obscure but the evidence strongly favours either an adaptation on the part of the original mechanocyte to the special conditions of culture and of survival in isolation, or, but with less probability, an initial selection of a cell type with properties somewhat different from any of the types discussed here. Until such changes occur, however, the cells within each family (amoebocytes, mechanocytes or epitheliocytes) apparently breed true, and do not readily change from one form to the other, though the possibility of such interchange is certainly to be considered. The transformation of epithelial cells from the kidney into cells of the generalized "tissue-culture" type certainly occurred spontaneously in a clone of such kidney-epithelial cells, and a new clone was derived from the mutated cells, and has itself continued to breed true (Parker, 1957) (Fig. 3.2 and 7.3). The cells of this clone, originally derived from epitheliocytes, may be compared in their morphology and growth pattern with the strain L fibroblasts.

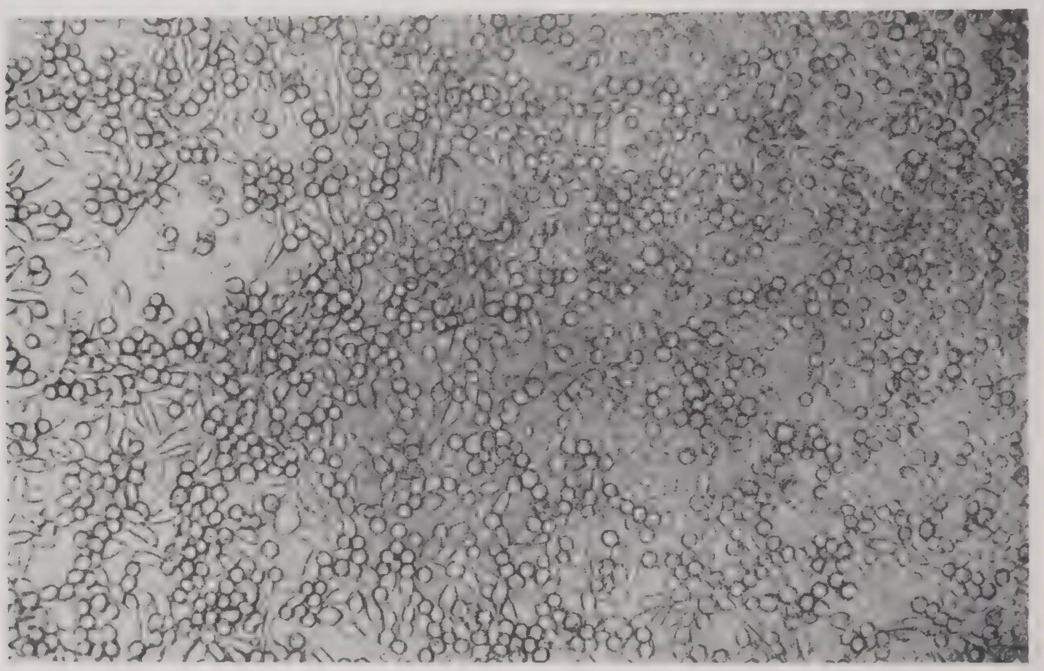

FIG. 7.2

Mouse fibroblasts (strain L) maintained in a synthetic medium for 210 days. Contrast these cells with those shown in Fig. 2.3. (Evans *et al.*, 1956.)

This property of breeding true within the family could be purely cytoplasmic, although in view of the adaptability of the cytoplasm to changed conditions, and in view of the apparently random distribution of such cytoplasmic elements as mitochondria at mitosis, a feature which contrasts so strongly with the elaborately equalized nuclear partition, it is perhaps more likely that these races of somatic cells in tissue culture are distinguished by nuclear characteristics. This does not necessarily mean anything so crude as a different number of chromosomes, though the counts of chromosomes in somatic cells are not sufficiently accurate or thoroughly known to preclude this possibility absolutely. All that it may mean is, that by some metabolic stimulus received during development, the nuclear pattern may be altered, i.e. the activity of one gene more than another may be allowed to predominate in a particular group of cells.

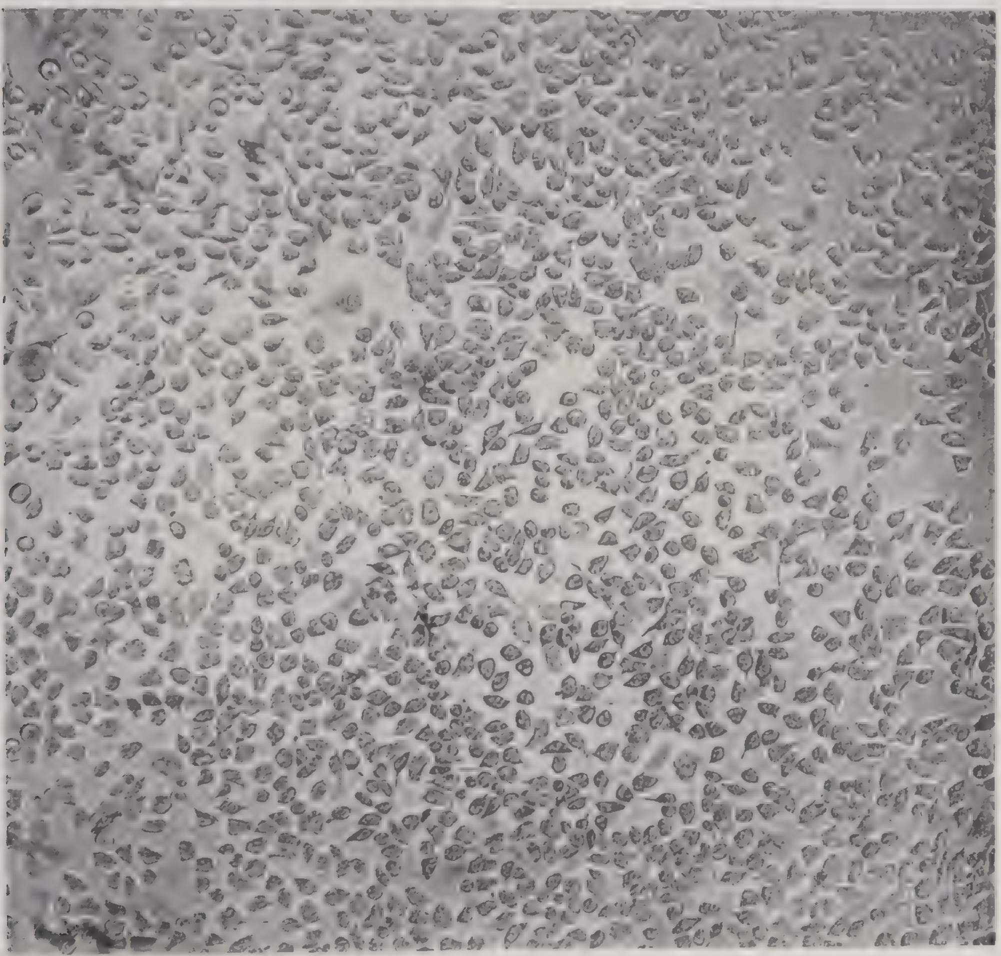

Fig. 7.3

Six-day old flask-culture derived from a nest of altered cells similar to those shown in Fig. 3.2. (Parker, 1957.)

Certainly there is now evidence that while in the early stages of amphibian development all the nuclei appear to be identical, nuclei in the endoderm of the late gastrula appear to be different. This follows from the observations that while nuclei extracted from cells in the early stages can be injected into, and can replace the nucleus of the unsegmented ovum and still allow normal development to take place, nuclei extracted from the endoderm cells of the late gastrula have lost this power and an increasing number of abnormal embryos results, when such nuclei are injected into enucleated but activated ova (King and Briggs, 1956).

Every histologist is familiar with the differences of nuclear form among the various somatic cells, some of which are illustrated in Fig. 7.4, and it is obvious that not all nuclei in the differentiated tissues are structurally identical, though in some early embryonic stages they may be shown to be physiologically identical or at least not irreversibly modified. The physiological meaning to be attached to these differences in appearance of the nuclei of different groups of cells has never been much investigated, yet it is clearly a very important phenomenon. Unfortunately, the detectable nuclear difference between the simplified epitheliocytes, mechanocytes, and amoebocytes of tissue cultures are not very striking, and in such matters as the number and size of nucleoli there is considerable variation within each group.

However, it might be worth investigating the relationship, if any, between the nucleoli and the degree of differentiation. It is conceivable that the nucleoli, as centres for ribose nucleoproteins, are concerned with organizing the synthesis of the cytoplasmic proteins and therefore more directly concerned with cell differentiation than are the chromosomes, which determine the species characteristics. If this were so, then the races of cells within the species would depend more directly on the behaviour of the ribosenucleoproteins than on the deoxyribosenucleoproteins. The temporary association of the ribosenucleoproteins with the chromosomes at metaphase and their subsequent dispersal into the cytoplasm during anaphase (Jacobson and Webb, 1951) may be a clue worth investigating from this point of view. Such a system could conceivably provide a basis for reduplicating the essential

1. Fibroblasts
2. Plain muscle
3. Columnar epithelium
4. Lymphocytes
5. Pancreas cells
6. Striated muscle
7. Liver cells
8. Nerve cell
9. Neuroglia cell
10. Neutrophil leucocytes
11. Monocyte
12. Megacaryocyte
13. Spermatocytes
14. Spermatids
15. Ovum

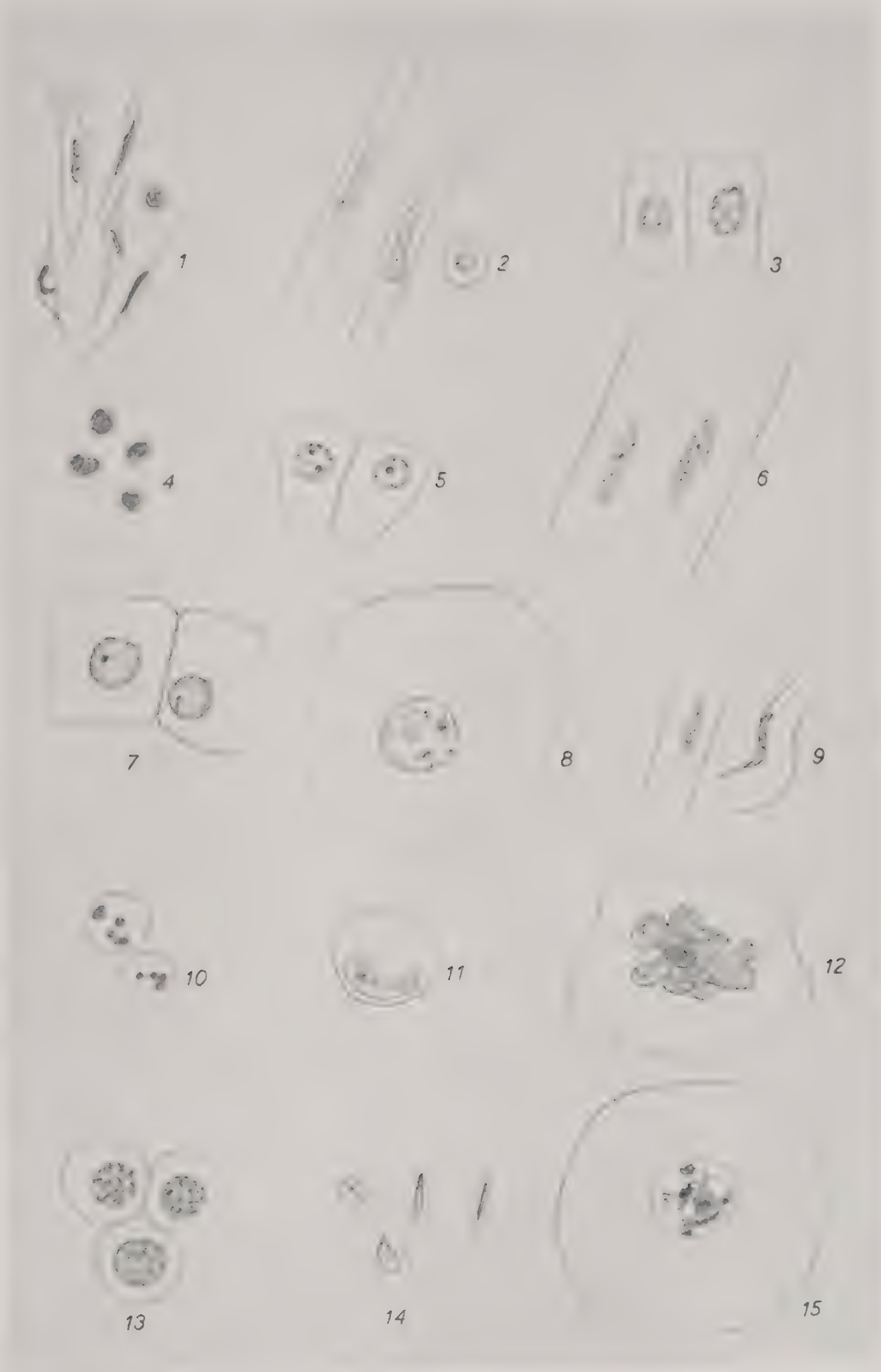

Fig. 7.4
Diagram to emphasize the different forms of nuclei in various somatic cells.

materials which allow the races of cells to breed true within the individual of any given species of animal.

There is said to be a difference in nucleoplasmic ratio (i.e. nuclear volume: cytoplasmic volume) between mechanocytes (0.138) and amoebocytes (0.054) (Strangeways, 1929), but in view of the variability in the cytoplasmic form and of the difficulties in assessing the ratio, little significance can be attached to this observation except as a pointer towards further and more searching investigations along similar lines with the establishment of a more meaningful ratio, e.g. $\frac{\text{Deoxyribosenucleoprotein}}{\text{Total protein}}$ or some similar measures of essential nuclear and cytoplasmic materials respectively. Perhaps the most sensible view to take is that the embryonic nuclei are capable of differentiating, probably under the influence of changes mediated through the cytoplasm, so that in future, they can produce a particular type of cytoplasmic organization, i.e. the activity of different sets of genes are called out by particular events in the cytoplasm, and once they have been called out, it becomes more difficult to suppress them and to evoke the activity of an alternative set of genes. Alternatively, the genes themselves could conceivably undergo modification in the somatic nuclei, perhaps at mitosis, when the ribosenucleoproteins become associated with them.

Thus, if it is supposed that in each race of cells certain genes control the cell activity, there may never be anything structurally very obvious in the actual chromosomes, which can be demonstrated to show that these dominating genes are distinct from the rest. To an observer from Mars, there would seem to be no visible distinctions between the men of the Kremlin and those of Westminster, which would be of sufficient magnitude to account for the macrophage activities of the U.S.S.R. and the epithelial behaviour of the British Commonwealth and the Nato powers. There are, however, historical and genetical factors at work in bringing about the diverse behaviour of the Russian and English social systems. Historical and genetical factors on a different scale are also worth investigating in cytology, as they must, in this sphere also, be concerned with the establishment of races, (i.e. of cells rather than of man), each with its specific type of behaviour.

Cell Types in Relation to the Germ Layers

Perhaps one of the more unexpected—and certainly one of the most significant—features of the existence of these three main families or races of cells, is the fact that the boundaries between them do not seem to be related to the dividing lines between the germ layers.

Epitheliocytes can emerge from ectoderm (skin), mesoderm (e.g. kidney), and endoderm (e.g. intestine), and so far as tissue-culture tests go, there is no general character by which to differentiate between them according to their origin. Epitheliocytes arise in culture from those tissues which are more or less directly connected with the original surface epithelium of the body, including of course the alimentary canal and its glands and diverticula, much of the urinogenital system and certain endocrine glands. Mechanocytes and amoebocytes, on the other hand, derive mostly from those cells which form the inner mass of cells in the body, i.e. those cells which leave the primary surface at a very early stage of development; these cells have been classed together as the "mesohyl" by Hadži (1949); the word means the "middle timber or substance", and it describes the place of origin of mechanocytes and amoebocytes far more nearly than does "mesoderm". Mechanocytes and amoebocytes can be fairly accurately described as emanating from the "mesenchyme" but this is not now a very satisfactory term, as it has been used with many different implications, e.g. it is often applied to the epithelial cells of the kidney rudiments; mesohyl is preferable.

This idea of surface and mesohyl cells, as the origin of epithelial cells and of the other cell types respectively, at first seems very simple; but the immediate question then arises from it as to why there should be two types of behaviour among the cells of the mesohyl and why all the mesohyl cells should not behave identically. In a general way all the epitheliocytes seem to behave very similarly in tissue culture, but the differences between mechanocytes and amoebocytes are certainly obvious and deep-rooted if not actually irreversible. Perhaps the behaviour of epitheliocytes *in vitro* should be studied in more detail. The plurality of types of epithelial cells *in vivo* (see p. 80) is very evident and could profitably be investigated in relation to possible differences among epithelial cells when living *in vitro*.

Before pursuing this question further, it will be preferable to discuss briefly the other groups of cells which do not conform with the three main types as described.

In the embryology of most vertebrates a portion of the surface epithelial cells are early marked off from the rest as neural plate tissue; this tissue sooner or later folds over, and eventually the folds join together longitudinally to produce a tubular form. Some cells, of course, fall just outside the neural plate and neural folds and so just miss being incorporated in this tube, but they appear to have acquired some of the characters of the neural tube tissue: such tissue is called the neural crest. Now it is from these parts of the embryo, the neural plate and the neural crest, that the majority of nerve cells, neuroglia and Schwann cells arise, i.e. it is from a region of the surface epithelium which at a comparatively late date in embryological development becomes specially modified. It is perhaps not surprising, therefore, that these nerve cells fall (as shown in Chapter 6) into a somewhat different category from the rest, and for the present, until the origin of the other categories has been more fully studied, it will be convenient to treat them as cells apart. They are clearly derivatives of epitheliocytes that emerge at a later stage in both ontogenetic and in phylogenetic history, probably as the result of the local changes in their environment, some of which are brought about by the proximity of the underlying mesohyl cells.

The neural crest itself is a tissue of great embryological significance, and one which has been the cause of much controversy; it is the nigger in the woodpile of the classical germ-layer theorists. From it there arise a number of interesting cell types which on classical lines are very difficult to reconcile with a rigid germ-layer theory. In the first instance the neural crest is essentially an epithelial sheet lying at the edges of the neural folds and, when the folds meet and the superficial epithelium closes over the surface, the neural crest cells are those which fail to become incorporated either in the neural tube itself, which is still fundamentally epithelial, or in the true epithelium of the surface. They are thus really epithelial cells forced out of the surface; in other words, they become mesohyl, though perhaps with special properties consequent on their location and on their late date of entry. It is therefore very relevant to the present discussion to note that from these ex-epithelial cells arise not only nerve cells, but cells of the pia mater, Schwann cells, cartilage cells and perhaps even bone cells and odontoblasts (Hörstadius, 1950) (i.e. mechanocytes) and also all the chromatophores, melanophores and dendritic cells of the body (which are all cells showing many properties characteristic of amoebocytes). As noted earlier, Schwann cells should perhaps be subdivided into two groups according to their behaviour, the spindly mechanocyte-like type being possibly distinguishable from the more diffuse type with irregular pseudopodia. A similar difference between the bipolar nerve cells of the dorsal root ganglia and the more diffuse type of the autonomic ganglia, may perhaps reflect a similar duality. Without delving into these unknown and rather complex regions at this stage however, it is clear that in the "epithelium" of the neural crest there are already the seeds of both mechanocytic behaviour and of amoebocytic behaviour; this epithelium, therefore, seems to contain the same sort of duality, or limited plurality, within itself as has been suggested as being inherent in nearly all the other epithelia of the body, when these are judged by the manner in which their cells may differentiate.

It is therefore of some interest to observe that the neural crest, which is such a very difficult tissue to incorporate neatly into any "classical" germ layer theory, is perhaps the very tissue which shows most clearly the manner in which the epitheliocytes, as soon as they leave the external surface, can acquire new properties and that such cells can then display one of two main lines of development. The final properties of the cells could be considered as variations on one of these two main themes –the mechanocytic theme and the amoebocytic theme—but the main problem obviously lies in the fact that there are again these two major lines of behaviour, and two only. Are epitheliocytes always divisible into two main groups, and if so, what is the nature of the difference between the groups? These seem to be the basic questions arising from this discussion.

Finally it should be emphasized that mechanocytes, amoebocytes and epitheliocytes are not the restricted property of the higher vertebrates. Similar behaviour has been noted also in the lower vertebrates in so far as successful culture experiments have been performed on their tissues; and there are reasons to believe that animals very much lower in the scale would, under similar conditions, produce the same general subdivision of behaviour amongst their main cell types.

Since the origin of vertebrates is shrouded in mystery, it is pertinent to ask at what level of animal organization the subdivision of the families of cells takes place, because, if it can be shown as a fairly general phenomenon in very much simpler organisms, then the details and uncertainties of the origin of vertebrates become more or less irrelevant to these problems of cell behaviour. Unfortunately, tissue culture has not been extensively practised on the invertebrates and it is of course difficult to know which invertebrates can be profitably regarded as giving direct information relevant to the stream of organisms whose evolution culminated in the vertebrate stem and which would be worth studying from this point of view. Extensive studies of amoebocytes in invertebrates have been made by Loeb (1920) who used the blood cells of the King Crab (*Limulus polyphemus*, Arachnidae) and their behaviour was found to be similar in many ways to that of amoebocytes of higher animals but was on the whole more plastic. It would appear that these cells are less irrevocably differentiated and are more nearly totipotent and capable of forming epithelial-like sheets and of assuming mechanocytic characters than are the amoebocytes of higher forms.

Liebman (1950) too, has studied the leucocytes of *Arbacia punctulata* (Echinoderm). Here again the cells are versatile and seem to be capable of giving rise to cells of other types, e.g. mechanocytes (fibroblasts), petaloid (? epithelioid) cells, amoeboid macrophages, and storage trephocytes. The plasticity of the amoebocyte is probably a rather general characteristic of these cells and reasons for this will be given later in considering the significance to be attached to this phenomenon.

Very interesting information is also available as the result of the study of some of the most primitive of all groups of metazoa, e.g. the Porifera, and there are indications from this study that amoebocytes, mechanocytes and epitheliocytes are indeed extremely primitive and fundamental as cell types and that they even precede the germ layers. In fact, there are indications that the basic patterns and forms of behaviour characteristic of these types may have originated even before the development of Metazoa. It will, however, be shown in the following pages that there is evidence that the basic differentiation is already present, though perhaps in a more reversible form, in the Porifera or sponges. This being so, the seeds of mechanocytes and amoebocytes may have their origin even in the Protozoa, and, as will be seen, there are reasons for believing that this is indeed so.

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CHAPTER 8

THE EMBRYOLOGY OF SPONGES AND THEIR CELL LINEAGE

WITH these ideas in mind it will be instructive first to examine the histology of certain calcareous sponges, and then to study in particular the manner in which these organisms develop and to see how the cells of the more mature sponge emerge from those of the embryo. Before doing this, however, it may be desirable to justify the choice of this material rather than any other. Especially is this necessary since it is probable that the sponges are an off-shoot from the main evolutionary tree. They have become specialized in their own peculiar way and have subsequently made very little progress in a direction relevant to the evolution of other phyla. It is thus not the purpose of this argument to show that the histology of adult sponges is directly related to that of vertebrates, though it will in fact become clear that there are certain rather interesting similarities, but what is intended is to use the developing sponge at the blastula stage, i.e. at the free-swimming larval stage, in order to emphasize certain properties of simple metazoan organisms. These primitive organisms, as we are probably justified in considering the blastulae and larvae of sponges, have much in common with the embryonic forms of many other phyla and it will be profitable to bear in mind for comparison the embryonic forms of certain coelenterates, platyhelminthes, nemertines, annelids, echinoderms and other invertebrates (Fig. 8.1). There are certain broad principles of organization which can be seen to be common to all these embryos, though the detailed embryology of each must, of course, as for other purposes, be treated as a special case with its own peculiarities. The methods of cleavage vary; the proportions of the cell groups vary; the methods of gastrulation differ and so on, but, basically, they all eventually form spherical blastulae or morulae. Embryos, it is true, may often be as specialized in their own way as are the adults into which they will develop, but nevertheless there are certain fairly simple common features which, if they can be extracted from the overlying complications and specializations, can be presumed to have some basic evolutionary meaning. The formation of the spherical and hollow blastula can, in this sense, be regarded as a critical stage in the evolutionary progress from the unicellular to the multicellular organisms.

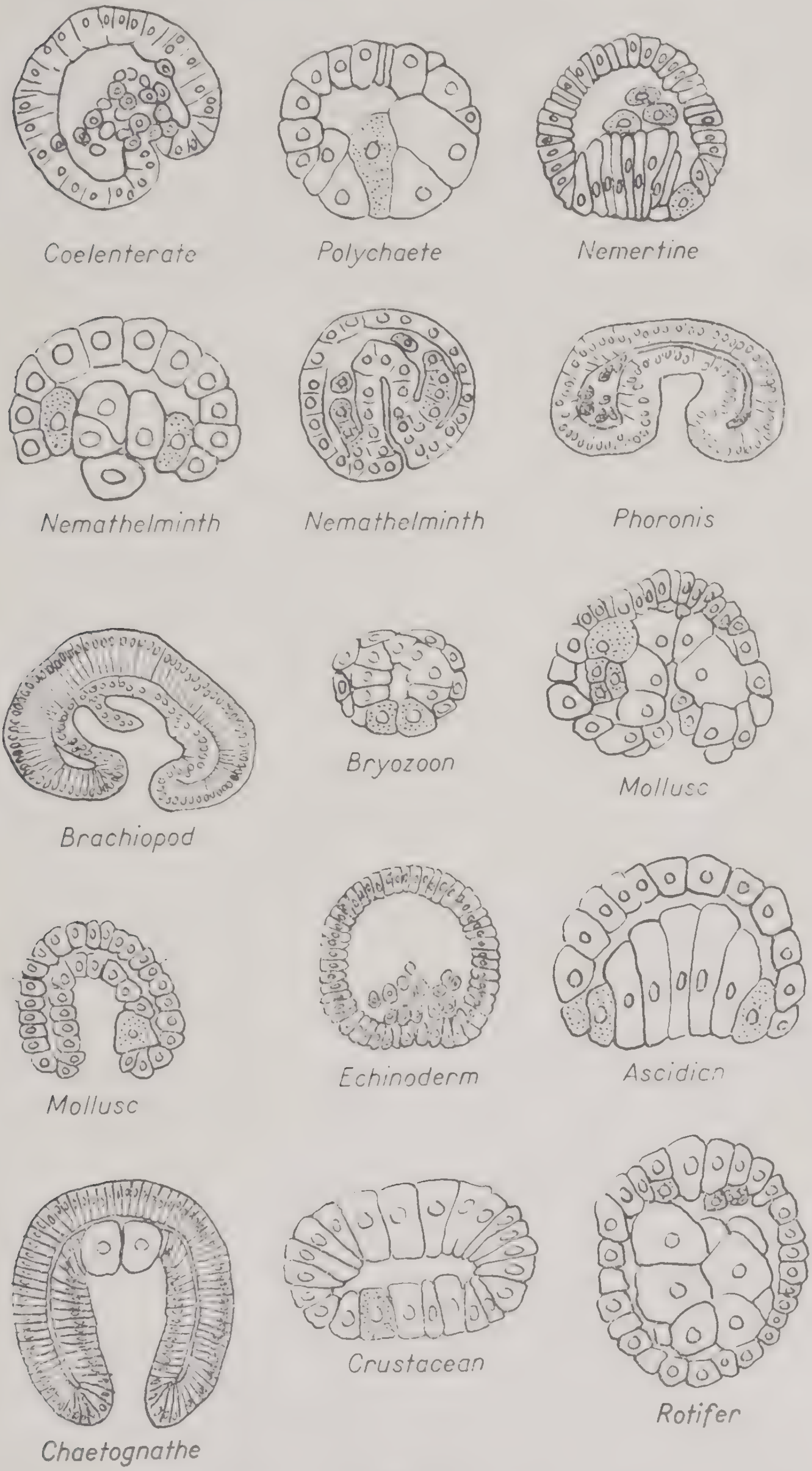


Fig. 8.1

Some early stages in development in different phyla. (After Dawydoff, 1928.)

It so happens that of all the organisms mentioned above the developing calcareous sponges offer peculiar advantages as organisms worthy of study from this point of view, though their embryonic history certainly has its complications. The cells are clear, and there is little disturbance from stored yolk. The organisms have great recuperative powers and can readily be subjected to experiment. Moreover, until the onset of the rather complicated and special metamorphosis of the later larva, the pattern of the free-swimming sponge larva gives the impression of being fundamentally simple and relatively free from the specializations which arise later and which are obviously peculiar to the sponges alone. However, it is worth noting that the manner in which the amphiblastula or larval stage is reached is not always simple even in sponges. In *Sycon* and *Grantia*, for example, where early cleavage takes place within the tissues of the parent sponge, a stomatoblastula is first formed; this then remodels, by a process not unlike that found in *Volvox*, and reverses the position of its cells before it becomes the free-swimming amphiblastula (Duboscq and Tuzet, 1937) (Fig. 8.2). This curious process may well be an

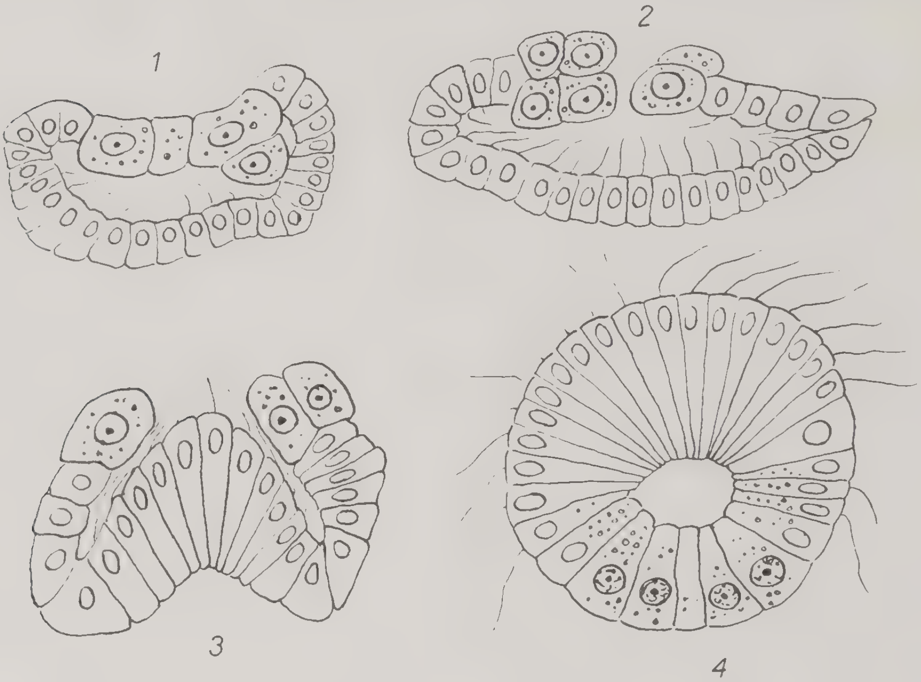


Fig. 8.2
Stages in the development of *Grantia* leading to the formation of the free-swimming amphiblastula (4). Stages 1, 2, and 3 are passed within the parent tissues.
(After Duboscq and Tuzet, 1937.)

adaptation to life within the parent tissues, and the reorganization is necessary before the organism is launched into the sea.

The essential features of blastula formation as seen in these simple organisms can later be recognized, though often with considerable modification, in the development of many other organisms. In other words, sponges seem to display the skeleton key, which in many other groups has had new and, from the point of view of the present discussion, irrelevant wards cut into it. Once the main key is recognized, however, it can be shown that it probably unlocks the basic pattern upon which most embryonic forms are constructed. This does not mean that higher metazoa are directly derived from sponges. All it does mean is that some primitive metazoan, from which eventually all the various phyla of both Parazoa and Metazoa have emerged, was probably—or at least passed through a stage of being an organism not unlike that which can be seen today in the free-swimming blastula of the calcareous sponge, for something akin to this basic pattern is of very widespread occurrence among embryos of the animal kingdom. Even among the different species of calcareous sponges, there are variations on the simple theme of exactly similar kinds to those which are found in the embryos of other groups, thus emphasizing the necessity for constantly striving to extract the key to the basic pattern and to eliminate the unessential specializations.

In the particular calcareous sponge, *Clathrina blanca*, the segmentation of the ovum is followed by the development of the blastula (Fig. 8.3). This is usually a hollow organism consisting of a single layer of cells. The majority of its cells are flagellated, but at the "posterior" end there are found, in this species at least (Minchin, 1900), two larger and non-flagellated cells: these are called the archaeocytes. In other species, as in *Sycon raphanus* (Fig. 8.4) and in the *Leucosolenidae*, most of the posterior half of the embryo at the corresponding stage is composed of non-ciliated, and phagocytic, granular cells; in the latter species there are also numerous cells, forming a band round the equator which are often very granular and seem to belong clearly neither to the anterior nor to the posterior cells. In sponges then there are, in many species, certainly two forms of cell behaviour evident in the early embryo, and in some species there may be a third, somewhat intermediate, kind.

During the next phase of development in *Clathrina* some of the cells in the anterior region lose their flagella (Minchin, 1900); they then break their attachment to the other neighbouring cells and sink inwards into the cavity of the blastula, thus forming the beginning of an inner mass of tissue which is again perhaps well called the mesohyl—

the central or inner mass or substance (see p. 127). At about the same time, earlier or later according to the species, the posterior and generally granular cells, or archaeocytes, multiply: then they, too, send representatives into the mesohyl. In these species, therefore, the mesohyl is built up from cells which have, at least temporarily, lost the "epi-

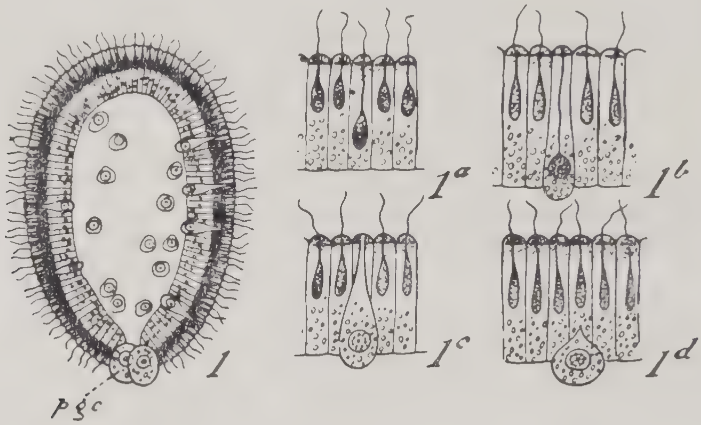


FIG. 8.3
Blastula of *Clathrina blanca,* showing the flagellated cells and archaeocytes, and the manner in which the flagellate cells may sink into the interior. (Minchin, 1900.)

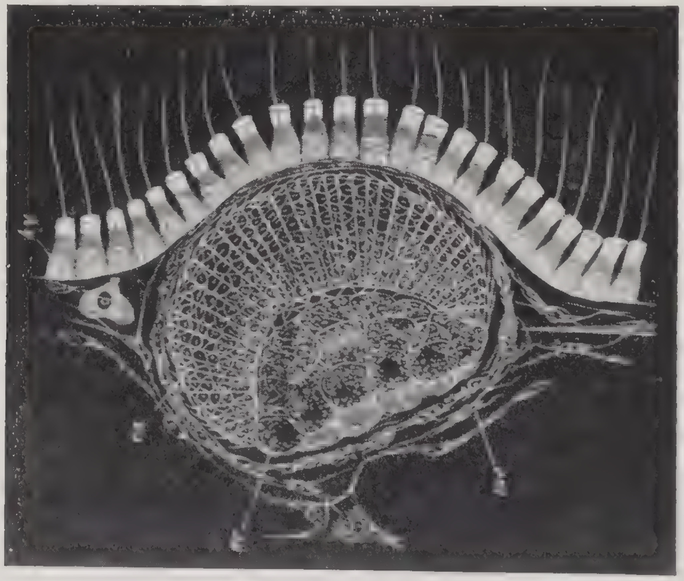


FIG. 8.4
Pseudogastrula stage of *Sycon raphanus* showing a clear distinction between two main types of cells in the larva, which still lies buried in the maternal tissues.
(From Balfour, after Schultze, 1885.)

thelial" characteristic of mutual cohesion; it is composed of cells which have broken away from the surface layer.

Moreover, the mesohyl cells are of two distinct origins, and this difference is further emphasized by the future fate of the two types. Of those derived from the archaeocytes, some develop into the amoeboid wandering cells of the body, i.e. the amoebocytes, including phagocytes and storage cells (thesocytes), while some remain as potential germ cells (tokocytes). Those derived from the flagellated cells contribute to the inner structure of the sponge by giving rise to several types of cell. They produce the spicule-forming cells (scleroblasts) and during the complicated system of larval metamorphosis, which, as already mentioned, is peculiar to the sponges and therefore largely irrelevant, they give rise to the cells of the dermal epithelium and to the contractile pore cells of the body wall (Figs. 8.5, 8.6). These latter cells eventually regulate the flow of the fluid into the cavity of the adult sponge, the flow itself being caused by the beating of the flagella of the collar cells (choanocytes) which line both the main cavity of the adult sponge and also the flagellated chambers in its walls. These choanocytes are the direct derivatives of the original anterior flagellated cells of the embryo and have never lost their connexion with the primary epithelium or entered the mesohyl except in a rather special manner during metamorphosis. The cell lineage in *Clathrina* is set out in Table 8.1. It is hardly necessary to point out at least the superficial similarity between the essential elements of the sponge and the main cell types in tissue cultures of higher animals.

The sponge cells, in *Clathrina*, are divisible into three main groups: (1) the true epithelial cells (the choanocytes); (2) the cells of the mesohyl derived from the ciliated cells. These cells transform into skeletogenous scleroblasts (cf. myxoblasts) (Figs. 8.5. and 8.8) and into the contractile pore cells (cf. myoblasts) (Fig. 8.5) while others may acquire a secondarily epithelioid type of behaviour to form the dermal epithelium (cf. endothelium and mesothelium (Figs. 8.5. and 8.6). (3) the amoeboid cells, also of the mesohyl but derived from the "archaeocytes" (Fig. 8.7). It perhaps should be made clear, however, that a somewhat different scheme for the classification of the cell groups has been drawn up by Duboscq and Tuzet (1937) as the result of their investigations on *Sycon* and *Grantia*, though their evidence does not seem to be entirely convincing.

In appearance and general behaviour the cells of these three groups can be directly compared with epitheliocytes, mechanocytes and amoebocytes respectively. Moreover, this general similarity can be further emphasized by the behaviour of sponge cells after isolation.

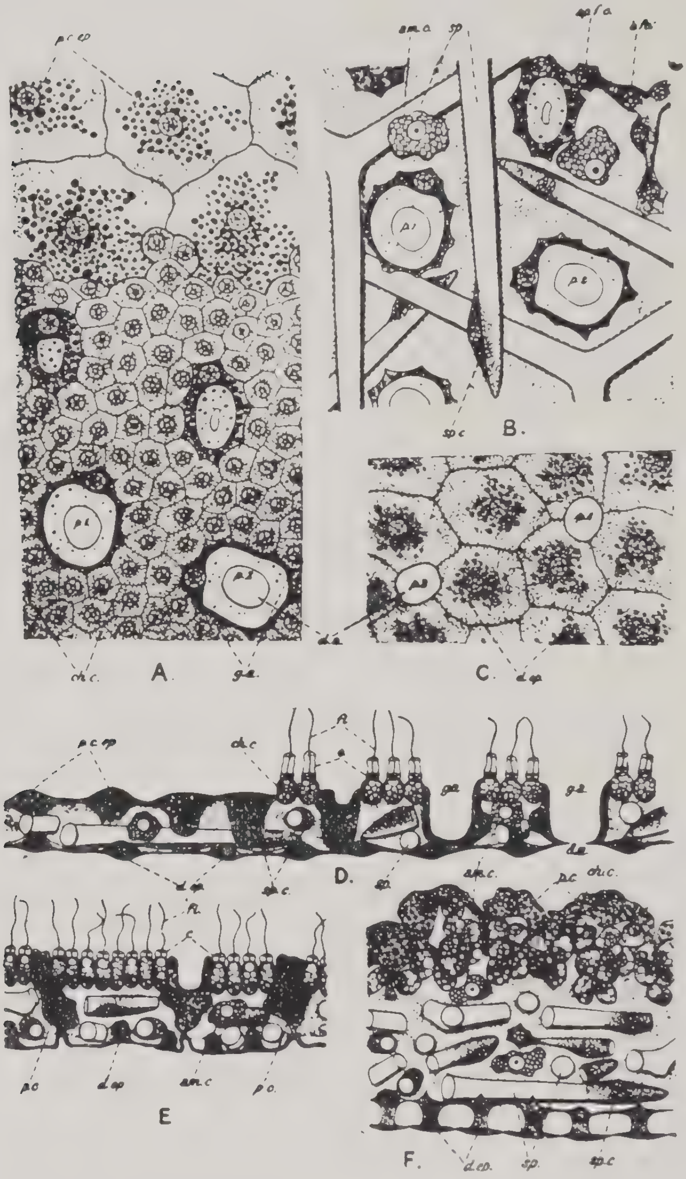


FIG. 8.5

Histology of the body wall of *Clathrina coriacea*, Mont.

A. Body wall seen from the inside near the oscular rim. B. The cells of the parenchyma in the same region. C. The same region seen from the outside. D. Body wall in longitudinal section (stretched). E. Body wall in longitudinal section (slightly contracted). F. Body wall in longitudinal section (very contracted). *am.c.* amoebocytes; *ap.f.c.* apical formative cell; *b.f.c.* basal formative cell; *c.* collars; *ch.* choanocytes; *d.a.* dermal aperture of pore; *d.ep.* dermal epithelium; *fl.* flagella; *g.a.* gastral aperture of pore; p^1, p^2, pores; *p.c.* porocyte; *p.c.ep.* porocytic epithelium; *sp.* spicule; cell or scleroblast. (Minchin, 1900.)

When pieces of calcareous sponges, e.g. *Sycon*, are pressed through fine silk into seawater, it is found that the cells which emerge and sink to the bottom of the vessel remain alive and active and can be easily studied in isolation (Fauré-Fremiet, 1932; Huxley, 1911; Wilson, 1907). Moreover, they eventually collect together into groups and under favourable conditions whole new sponges may emerge from the fragments. At one time this sort of activity seemed to be peculiar to sponges, but it is now known to occur elsewhere; indeed, something very similar happens when the cells of organ rudiments of chicks and mice, e.g. limb-bud, kidney or pituitary, are separated by treatment

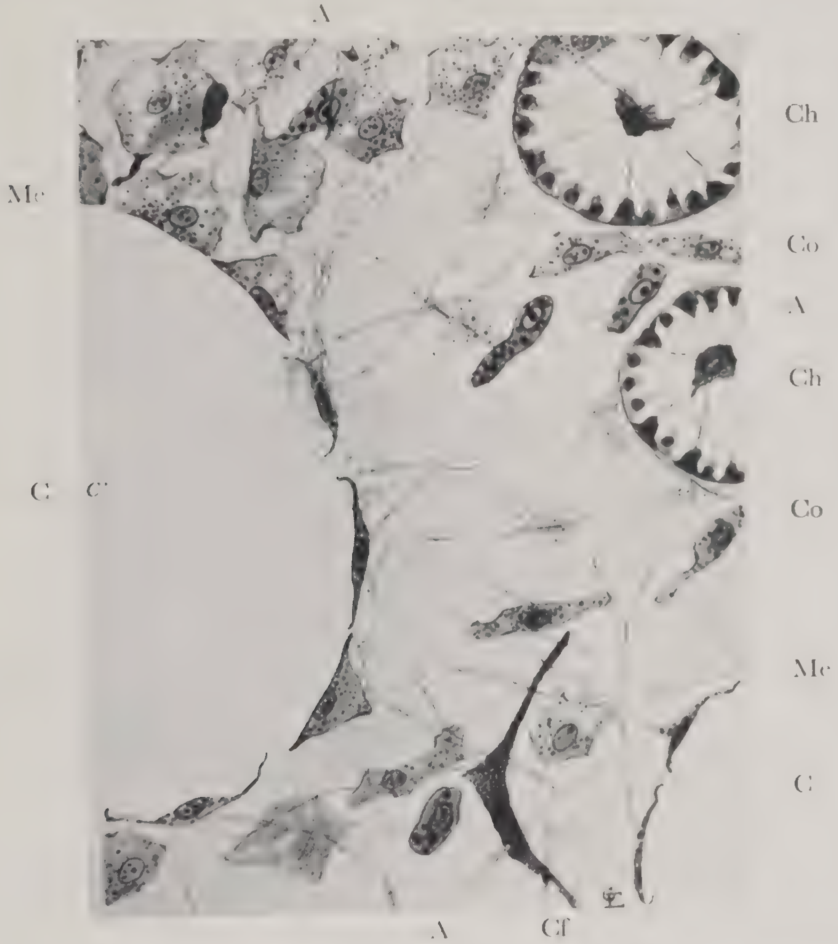
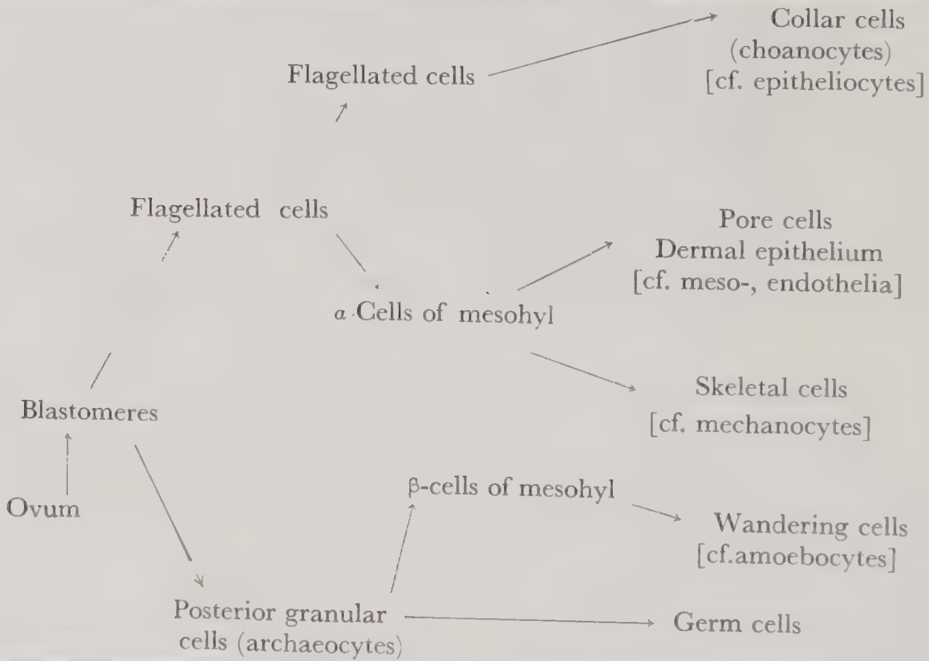


Fig. 8.6

Drawing of a section through the wall of a sponge (*Ficulina ficus*) showing flagellated chambers with choanocytes (Ch). Surface or dermal epithelial cells (Me) lining the canals (C). Scleroblasts or collencytes (Co), amoebocytes (A) and fuchsinophil cells (Cf) occur in the substance of the wall. The groundwork of collagen fibres is clearly shown. (Fauré-Fremiet, 1931).

with trypsin in a Ca^{++}- and Mg^{++}- free salt solution and then allowed to reorganize in tissue culture (Moscona, 1952a and b). Here again the isolated cells regroup themselves and reorganize into something closely similar to the original tissues. If the freshly isolated cells of the sponge are examined, particularly with the help of the speeded-up cine-film, their movement and behaviour can be readily studied. Under these conditions the collar cells first modify their collars, which may break up into several filiform motile pseudopodia, and then the flagella also may disappear, but whatever happens in this way the cells show a great tendency to stick together and so to form clusters. This adhesive quality is of course an essential property of the cells of the early embryo and a characteristic of all epitheliocytes, and when cells leave the surface and enter the mesohyl they must have lost their capacity to stick to their neighbours. Choanocytes, after dissociation, may show amoeboid movement at the proximal end of the cell and the other end of the cell may still have a flagellum and produce pointed pseudopodia. Cells of this type thus appear to be polarized structures, one end being different from the other, and they also have a tendency to adhere to other cells of the same type. Very similar behaviour has been seen among ciliated cells separated from amphibian embryos (Holtfreter, 1947) (Fig. 7.1).

TABLE 8.1



Cell lineage of *Clathrina blanca* (modified after Minchin, 1900).

In the sponge embryo, the flagellated cells (polarized and adhesive) give rise to the mechanocyte-like cells or scleroblasts and it is therefore interesting to find in the sponge pulp that there are some cells which move with a definite polarity but have no flagella, though their anterior pointed pseudopodia are often flexible and may bend at right angles and come and go in somewhat random fashion. Such cells are usually rare in these preparations but this may be due partly to the fact that many of them probably remain, as they normally are found, attached to the spicules and spongin fibres (Figs. 8.5. and 8.8), and these are mostly held back by the silk. In contrast to both these types, choanocytes and scleroblasts, there are always present numerous large cells, generally with a single conspicuous nucleolus in a spherical nucleus, which wander about with random amoeboid movement, throwing out long branching pseudopodia of quite irregular form (Fig. 8.7). These cells show little tendency to aggregrate in clusters though they may form giant cells (Fauré-Fremiet, 1932) (Fig. 8.9), and when they become involved in a cluster of choanocytes they still retain their independence and may leave the group and wander away on their own. These are clearly the amoebocytes and the potential germ cells; and, in the form of their pseudopodia, in their ability to store trypan blue, and in many other features, they bear a close resemblance to the amoebocytes of higher vertebrates (Figs. 5.1 and 5.5). In the sponge, some of these cells may contain granules and secretions of various sorts, and no doubt some of these variant forms are peculiar to sponges

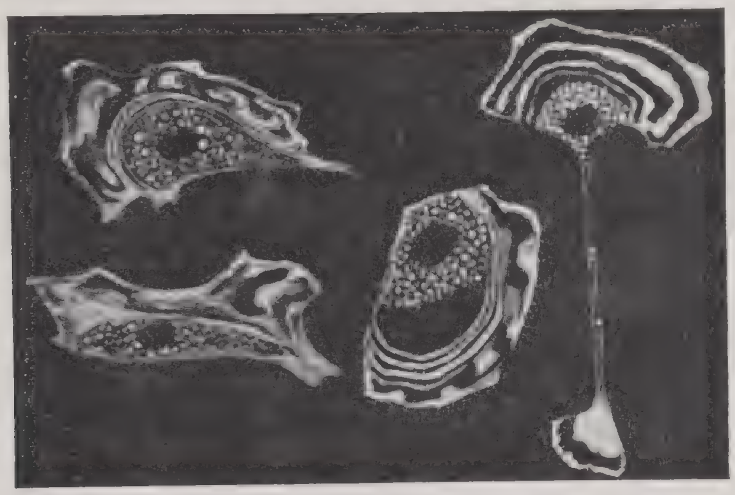


Fig. 8.7

Amoebocytes (archaeocytes) of *Ficulina ficus* as seen by dark field illumination. Compare these cells with those shown in Fig. 5.1. These cells segregate trypan blue. (Fauré-Fremiet, 1932.)

in general, and even to the species in particular. These variations do not, however, detract from the basic similarity to other amoebocytes.

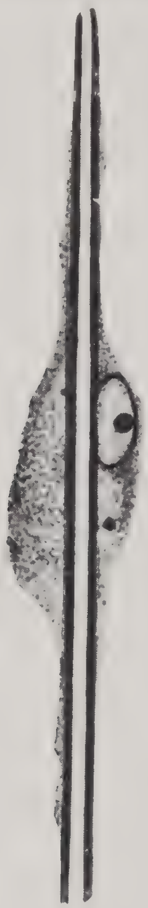


Fig. 8.8
Scleroblast attached to a spicule. (Fauré-Fremiet, 1931.)

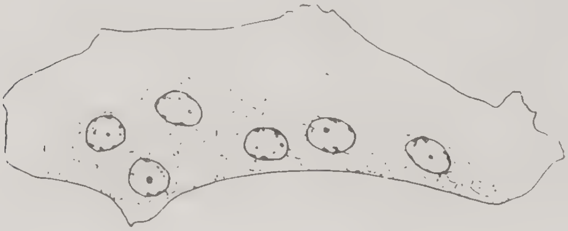


Fig. 8.9
A multinucleate giant cell formed by fusion of amoebocytes from the sponge *Ficulina ficus* L. (Fauré-Fremiet, 1932.)

In the sponges, therefore, there are some polarized cells which normally show an essentially epithelial type of behaviour and adhere to each other strongly (the choanocytes); there are some cells which are also polarized but which are more independent of each other and behave like mechanocytes, and these are particularly connected with the sponge fibres and spicules (scleroblasts); thirdly, there are the large random amoebocytes, completely independent and with no obvious polarity. The interest in the cells of the sponge mesohyl must deepen when it is realized that the supporting fibres of the sponge (spongin) which are thought to be formed by the mechanocyte-like cells (scleroblasts) have recently been shown by electron-microscopy (Fig. 8.10),

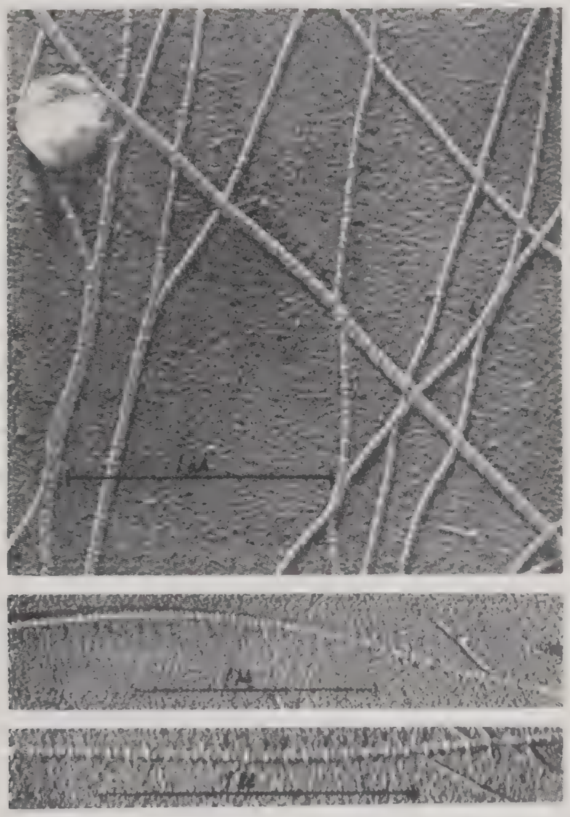


Fig. 8.10

Electron-micrograph of spongin "A" fibres from a sponge showing 600-700Å axial periodicity as in collagen, with 3 or 4 intra-period bands. (Gross, Sokal and Rougvie, 1956.)

X-ray diffraction and chemical constitution to be collagenous in nature and to lie in a matrix containing many of the same amino sugars, carbohydrate and polysaccharide elements as the mesenchyme of vertebrates contains (Gross, Sokal and Rougvie, 1956). It is thus tempting to think that the "connective tissues" of the sponge are produced in much the same way as the connective tissues of higher organisms.

Epithelial cells of certain types; e.g. the flagellate type, may also be able to produce amino sugars, polysaccharides and their derivatives, externally to themselves. For example, it is relevant to bear in mind that if the early blastulae of certain sea-urchins are stained for mucoproteins the fluid within the blastocoele gives a positive reaction (Monné and Härde, 1950), even though, at that stage, the blastula is composed of nothing but mutually adherent flagellate cells not yet enclosing any "mesohyl" cells. Moreover, the chitin of arthropods is an epithelial "matrix" and the "terminal bars" of various columnar epithelia in vertebrates are probably of somewhat similar nature. "Ground substance" may thus not be the exclusive prerogative of mesohyl cells, though it is a feature of cellular activity which becomes very highly developed by at least one main class of such cells.

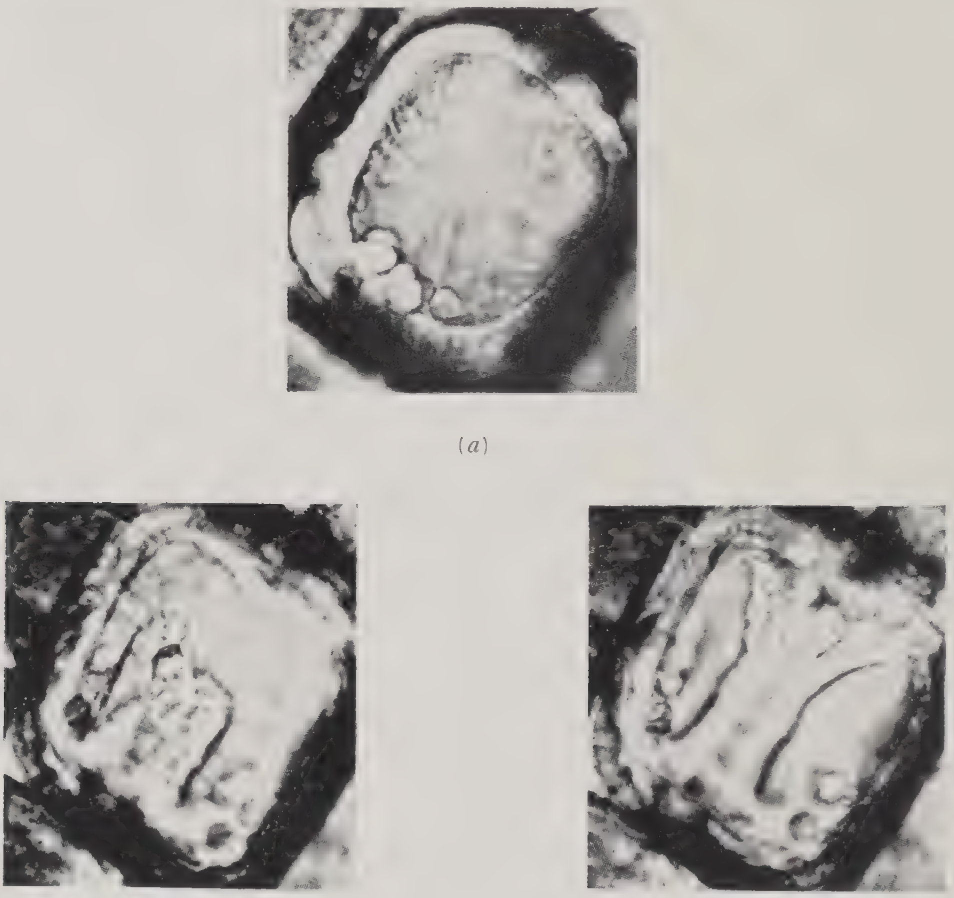
In the embryos of some sponges there is a sharp transition between the cells of the flagellate group and those of the posterior zones, and in these species the change from one type to the other is sudden and "all or none". In other sponges, e.g. *Grantia,* a very similar general arrangement of the cells occurs but there may not be such a sharp transition, so that cells of intermediate form are more or less numerous. In other words there is a rather steep antero-posterior gradient.

Extending far beyond the confines of the Porifera, an antero-posterior gradient is, indeed, almost universal in embryos and has been well recognized as the primary axial gradient. It can be detected in some species by different vital-staining reactions of the animal and vegetal poles (Spek, 1933, 1934, 1938) though the interpretation to be placed on these differences is not immediately obvious. In other species it can be demonstrated by differential susceptibility to poisons, by the pattern of gastrulation by invagination, by the zoning of pigments, mitochondria or yolk granules, and by a variety of other methods. It thus appears that in sponges there is certainly a very clear demonstration, perhaps an accentuation, and even a pointer to the actual origin, of something which is of more or less universal occurrence in the animal kingdom. For this reason, the sponges, being among the most primitive of multicellular organisms, may throw light on the meaning to be attached to the gradient, since in some of them the gradient is so sharp as to amount to an almost "all or none" distinction between anterior cells and posterior cells. If it were possible to come to some conclusions as to the cause of the difference between these cells in sponges, then this would presumably be relevant to the understanding of the meaning of axial gradients as a whole and perhaps eventually to the proper interpretation of the significance of ectoderm, endoderm, mesoderm, mesenchyme and the like, since, by and large, these are derived from different regions of the embryo, that is to say, from cells occupying different positions along the gradient.

As pointed out earlier, each embryonic history is likely to be a law unto itself, certainly in respect of detailed cellular arrangement, and the cells in the more complicated embryos may move around during development and even become inextricably intermingled, thus often obscuring the primary gradient and bringing others into prominence. In sponges there is probably a very real difference between the anterior and posterior cells which develops at a very early stage. The primary blastomeres must therefore very quickly acquire differences. Should this difference be decreased for any reason, i.e. should the gradient only be developed slowly, as it more often is, then "intermediate" cells are likely to be more numerous and the separation into definite types made that much the more difficult.

The situation in sponges is paralleled to some extent in molluscs where, in the young embryo, there can be traced an epiblast and also cells of two distinct forms contributing to the inner mass. Even more interesting, however, is the situation in echinoderms where the formation of the inner mass has recently been followed in motion-picture studies by Gustafson and Kinnander (1956) and the earlier accounts of the process fully confirmed. The first cells to contribute to the inner mass are cells which become rounded and "archaeocyte-like" and which enter separately from the vegetal pole. Then follows a true invagination of those cells which have moved posteriorly to the vegetal pole and have thus replaced those cells which have already entered the inner mass. From this invaginated segment there stretch out into the cavity to reach the cells of the animal pole, a second invasion of spindle-shaped cells whose processes and behaviour appear to be different from those of the cells of the first invasion (Fig. 8.11). These later cells are much more fibroblast-like or mechanocyte-like in appearance, and by their contraction they shape the invaginating endoderm (Dan and Okazaki, 1956). They are derived from cells which in the early blastula would be flagellated cells lying rather towards the vegetal pole. In the echinoderm, then, the development of the mesenchyme again suggests the presence of cells within it of two distinct types, and these two types resemble, superficially at least, the two types seen in sponges. Since it is generally believed that the situation in echinoderm embryos is probably more closely related to the situation in vertebrate embryos than is that in the sponges, the essential duality of the embryonic mesenchyme or mesohyl is certainly brought nearer home.

This being so, it will be advantageous to turn again to the more primitive situation, namely to those sponges in which there is a sharp separation between flagellated anterior cells and amoeboid posterior cells. As Metschnikoff pointed out in 1886, the differences between the anterior and posterior cells of such organisms as the sponge embryos must lie at the root of the whole problem of differentiation, and in these organisms the cells are conveniently susceptible to experiment and investigation. Moreover, it is pertinent to enquire whether this simple separation of the cells of the blastula into two main classes may not be the outcome and consequence of the essential evolutionary step


(*a*)

(*b*) (*c*)

Fig. 8.11

Formation of mesohyl in echinoderm embryo. (*a*) Invasion of posterior amoeboid cells (*b*) Invagination of posterior pole, and second invasion by mechanocyte-like cells originating from the invaginating cells. (*c*) Invagination connected to anterior pole by mechanocyte-like cells. Cells of original invasion still present towards posterior pole. (Gustafson and Kinnander, 1956.)

which yielded the first really viable Metazoa in their evolution from the Protozoa. Are the cells in the two classes in some way complementary to each other? Is there a sort of symbiotic relationship between them?

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CHAPTER 9

PROTOZOA AND THE SPONGE EMBRYO

JUST as the evolution of vertebrates from some invertebrate ancestor is still a major problem and may remain for ever somewhat of a mystery, so the evolution of Metazoa from "simpler" organisms has been the subject of much theorizing and speculation: nor is it yet solved. Nevertheless, there can be no doubt that the evolution of Metazoa must have occurred from unicellular organisms, and the latter must therefore be scrutinized in order to establish which of them are most nearly related to those organisms which gave origin to the multicellular forms. This scrutiny must be concerned not only with the outward forms of cells, which in existing Protozoa may be the result of some irrelevant specialization, but should also delve into the metabolism, behaviour and general organization of the cells and should consider such properties as polarity, adhesiveness, phagocytic powers, ionic and water equilibria, enzymic content, ability to produce or metabolize particular chemical substances, and all suchlike characteristics, but always discarding such properties as appear to be the result of immediate specializations for local conditions, unless they can be taken as evidence for the recrudescence of some atavistic character, or as the beginning of some feature which subsequently becomes widely adopted.

It has frequently been pointed out that the collar-cells (choanocytes) of sponges bear considerable resemblance to the free-living choanoflagellates among the Mastigophora (Fig. 9.1). Moreover these Protozoa themselves often produce colonial forms and it has been suggested that sponges are the ultimate outcome of such attempts at a communal existence. This may be correct, but it ignores the important fact that the sponge colony contains several types of cells within it and even the sponge embryos show two visibly different types of cell. This latter observation suggests derivation from two types of cell or, more probably, from a cell with two types of behaviour inherent within it. These types of behaviour may be either spatially or temporarily separated. The former may occur in strongly polarized cells: indeed, the choanocyte of the sponge has something of the necessary quality in that one end is essentially flagellate while the other is amoeboid. Many flagellates and some ciliates among the Protozoa show a similar and definite spatial polarity. Among the protozoan flagellates and amoebae, however, there also occur species (e.g. Mastigamoebae) in which the behaviour of the organisms changes radically from time to time, in that they can exist as amoeboid cells or as active free-swimming flagellates according to external conditions. Such "Jekyll and Hyde" cells may therefore yield important clues as to the biological origin of these two manifestations of behaviour and as to the significance which is to be attached to them. Moreover, this lability in behaviour would be expected to lead to further development or evolution along one or more of several lines since it could theoretically allow colonization of two different environments.

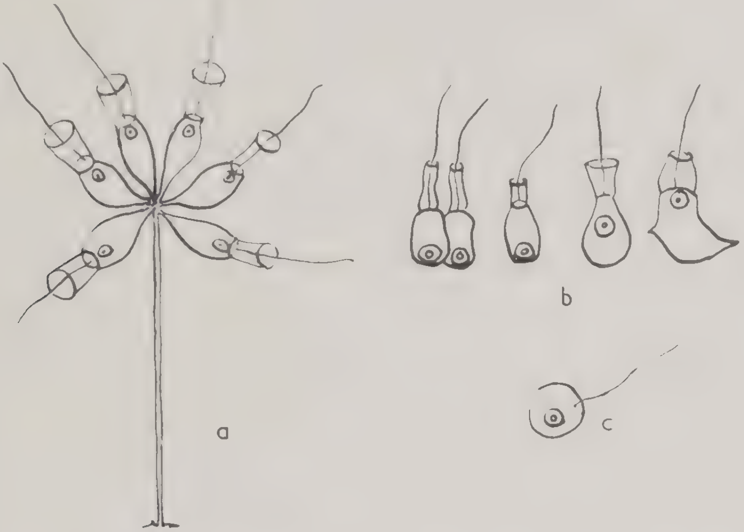

Fig. 9.1

Comparison between the choanoflagellates and the choanocytes of sponges. (*a*) *Codonosiga botrytis* (Mastigophora). (*b*) Choanocytes from sponges. (*c*) A choanocyte after loss of its collar, as seen in cell suspensions from sponges.

The two types of behaviour are well seen in such organisms as *Naegleria* (*Dimastigamoeba or Vahlkampfia*) *gruberi* (Schardinger, 1899) and *Histomonas meleagridis* (Tyzzer, 1934) (Figs. 9.2 and 9.3). In the first of these a flagellate phase readily interchanges with the more commonly found amoeboid phase. In the closely related *Tetramitus rostratus*, the flagellate phase is sometimes the more stable. A similar change of behaviour is not uncommon in other species also, particularly when the formation of gametes and reproductive processes are involved.

If it be supposed, for the sake of argument, that the primitive protozoan ancestor of *Naegleria* were a flagellate, then there would inevitably be certain limitations on its behaviour and on the environ-

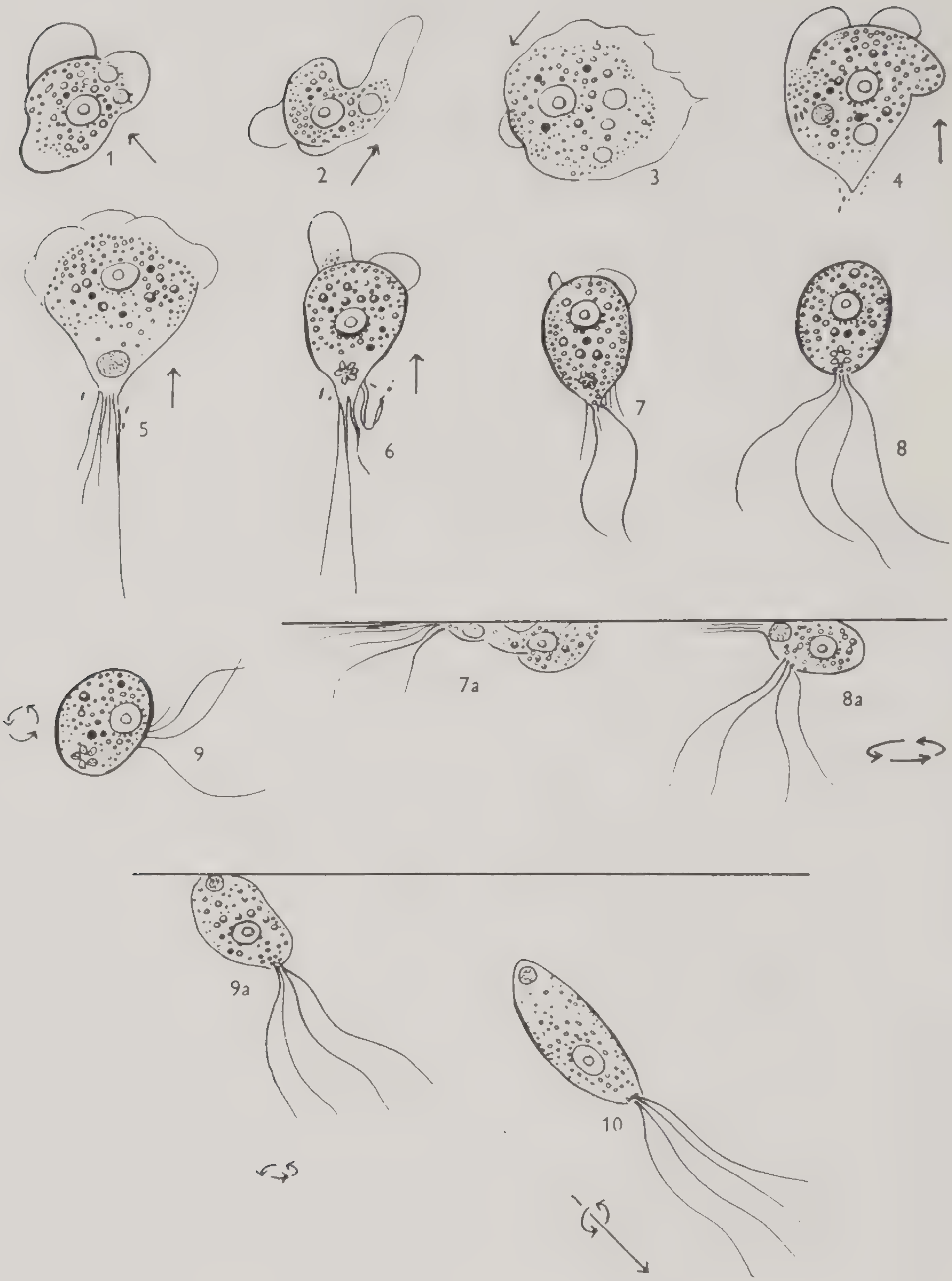

FIG. 9.2

Diagrammatic representation of the change of form of *Naegleria* when the organism is placed in distilled water. The arrows indicate the direction of motion.

1–3. Amoeboid form. 4. "Polarized" form. 5, 6. Filiform pseudopodia present. 7–10. Acquisition of flagella.

7a–9a are schematic of how events would appear if seen from the side when the amoeba leaves the surface of the coverslip. (Willmer, 1956).

ments which it could occupy. A very similar argument would apply if the primitive protozoon were amoeboid. Which of the two forms is "hen" and which is "egg" in this situation therefore makes very little difference to the following argument, which will be an attempt to trace out the probable consequences of such limitations of behaviour.

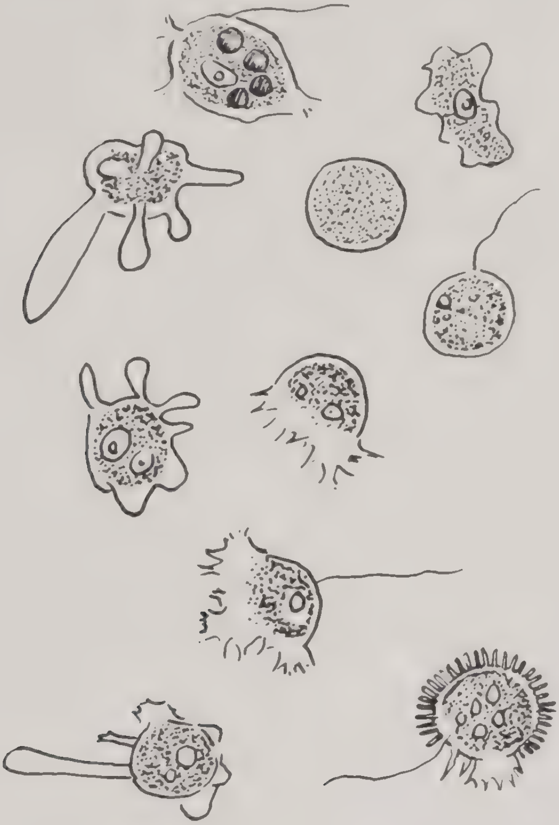

Fig. 9.3.
The various forms of *Histomonas meleagridis*. (After Tyzzer, 1934).

A flagellate protozoon has considerable powers of locomotion so that it may move from one environment to another if and when the first surroundings become unsuitable. Many flagellates, if they are not capable of photosynthesis, depend for their energy supplies on the extraction of materials dissolved in the fluid in which they are swimming, i.e. they lead a saprobiotic existence. This mode of life is manifestly somewhat precarious and while it may be generally suitable for those species which always live among decaying organic matter or in the body fluids of higher animals, it must inevitably come to an end when the food supplies are exhausted. Among the most primitive organisms, i.e. those existing before the days of higher animals and plants, it must have been still more precarious and, moreover, some sort of cyst or resistant form would be essential in order to tide the organisms over periods of interruption in the food supply. Probably to minimize such risks, some flagellates acquired chlorophyll and so could obtain their energy for the photosynthesis of some of their necessary metabolites by absorbing light energy from the sun. Examples of both modes of existence, saprobiotic and holophytic (photosynthetic), are numerous among existing flagellates and often both types of behaviour may be found in one species; i.e. there are some flagellates which develop chloroplasts when in the light but which can also carry on without them in the dark, if they are given the right nutritive conditions. Most flagellates normally lead an independent existence and, though their cells are strongly polarized, they show no tendency to aggregate together. Should such chlorophyll-containing flagellates, however, acquire a capacity for mutual adhesion and thus form colonies, organisms similar to *Gonium* or *Volvox* would presumably result. This latter blastula-like organism is apparently composed of only one type of cell, but it has two strings to its bow in the sense that it can absorb certain food materials ready-made from its surroundings and it can also use photosynthesis to manufacture them. Moreover, it is mobile and can change its environment. As another possibility the simple colourless flagellate may acquire some mechanism for obtaining solid food and digesting it within its body, by developing a sort of mouth and pharynx and modifying either the flagellum or the body surface, or both, in such a way as to direct food particles into the mouth. This type of feeding has been adopted, for example, by the choanoflagellates and by many ciliates. In the latter, some of the cells which have specialized along these lines have produced very large polarized cells with many nuclei, but true colonies do not seem to exist as such. On the other hand, the colonial forms of the choanoflagellates are well known (e.g. *Codonosiga botrytis*, Fig. 9.1) and have some features in common with the sponges. On the whole, however, this is likely to be a somewhat restricted mode of feeding and the particles which could be collected by the flagellar apparatus in this way would mostly be small, and thus the total supply might be somewhat limited.

A more efficient way of collecting solid food particles when they are abundant, is probably that adopted by the amoebae, namely, to creep along the available surfaces, such as the bottom of the pond, and engulf or phagocytose those particles, e.g. bacteria etc., which have food value and which are normally abundant on the bottom or are continually settling thereon. An organism with such a mode of existence is almost certainly assured of sufficient food, provided that the whole environment does not become unsuitable through drying up, high acidity, alkalinity, high salt content or other adverse conditions. Even when food is scarce, amoebae may persist for a time on the stored material for which the amoeboid form is perhaps rather more adapted than the stream-lined flagellate form. If, in addition, the amoeba also had the power to become motile by the acquisition of flagella or cilia, the evil effects of adverse conditions could be largely minimized and the advantages of the flagellate or ciliate mode of existence could be reaped. A cell, then, which could reversibly change from the essentially saprobiotic existence of the free-swimming flagellates to the phagocytic way of life of the amoebae, would be likely to be a successful competitor in colonizing new environments by being able to make the best of both worlds. Now there are, as indicated above, actual living examples of such organisms which can change from flagellate to amoeboid forms and vice versa, and their study well repays investigation. *Naegleria gruberi* is a remarkably clear example (Fig. 9.2). This organism is normally an amoeba which lives in the soil and presents no unusual features. It is strongly phagocytic and engulfs bacteria freely. It normally moves like most other amoebae, i.e. by means of lobose pseudopodia which may take origin from almost any part of the cell surface. Sometimes the cells become more like the "limax" type of amoeba with most of the pseudopodial activity at the anterior end and a more or less well-developed "uroid" at the tail end. This change is particularly liable to happen when the amoebae are placed in a more than usually watery medium. In fact, when *Naegleria* cells which have been cultured, for example, on a beef-extract-agar medium are washed in distilled water and then left in pure water, they very quickly undergo a definite series of changes. The whole cell first becomes fan-shaped, with the broad end anterior, and visible signs of an antero-posterior axis develop. Under these conditions the contractile vacuole, which previously had no fixed position, becomes definitely located at the posterior pole, and its whole cycle takes on a regular pattern, each filling phase being initiated by the appearance of about six small vacuoles which ultimately coalesce and then expel their contents. The frequency of contraction seems to remain roughly constant. At about the time when the contractile vacuole moves to the posterior end of the cell, fine filiform pseudopodia, much like those seen on the choanocytes of sponges when isolated in sea-water, appear at the posterior end. This intermediate state, neither flagellate nor fully amoeboid, is of some interest in connexion with the intermediate cells already referred to in the antero-posterior axis of some sponge and other embryos. The polarization of the cell with lobose pseudopodia at one end and with filiform pseudopodia at the other, is also very significant and has its counterpart in certain epithelial cells of higher animals.

These pseudopodia may show movements which are, at first sight, somewhat reminiscent of flagella; they may behave very like the filiform pseudopodia of the choanocytes and bend at right angles, thicken up, or produce minute undulating membranes: often they collapse back into the body of the amoebae only to be replaced by new processes of similar character. Later, among these processes, there appear one or more well-defined and genuine flagella with quite typical flagellar movement. At first, these processes are short and beat slowly, but they soon lengthen and their beat quickens. From one to four flagella can then be seen in active motion and attached to the posterior end of the cell. In stained preparations, it can be seen that there is a basal granule at the point of origin of each of the flagella. Very soon after their formation the flagella move their point of origin over the surface of the cell. They move away from the posterior pole, which for a time remains the place where the cell maintains its attachment to the surface upon which it was creeping, and travel towards the opposite pole. The nucleus also appears to move slowly away from the posterior pole with the flagella, but the contractile vacuole remains near the point of contact between the cell and its solid support. Eventually the flagella can be seen to beat freely at the pole of the cell opposite to that occupied by the vacuole. Their movement becomes more and more rapid till the now flagellate organism begin to rotate around its point of attachment, slowly at first then faster and faster, till this bond with *terra firma* finally yields and the organism swims freely away to pastures new. During this radical change from the amoeboid form to the flagellate form, there is clearly a great change in the character of the cell's surface. The whole cell becomes more oval or pear-shaped and pseudopodia cease to be formed. The cell becomes "stream-lined." In the fully differentiated flagellate form it should be noted that the flagella are situated at one pole and the contractile vacuole remains at the other (Willmer, 1956). This type of arrangement of the cell organelles is important; it may be expected to leave its trace in any organisms or cells which may have evolved along similar lines, and which may owe their origin to such organisms as *Naegleria*.

Here, then, in *Naegleria gruberi* is an organism which is, as it were, getting the best of all possible worlds. It can take advantage of copious food supplies by phagocytosis when in the amoeboid form and its growth in the amoeboid form is sometimes prodigious. When, however, the environment is flooded with water and food is scarce it can, in something over an hour, and generally less than four hours, assume the flagellate form and swim off in search of fresh food supplies or of new environments. In cultures, the flagellate stage does not generally last for many hours and can be shortened by the addition of salts to the medium. The organisms then settle down again as amoebae. This perhaps bears out the suggestion that the flagellate form is, for this organism, the less economical mode of life for everyday activity; it is, however, not yet possible to say for how long the flagellate phase might last if the fluid medium were to contain no salts, but with adequate supplies of the necessary soluble nitrogenous food substances, and to remain free from bacteria. Bacteria-free cultures of these organisms do not appear to have been produced as yet and little is therefore known of the minimum requirements for the saprobiotic(?) flagellate form of existence. It is, however, interesting that a related form, *Tetramitus rostratus*, which has a definite "mouth" in the flagellate form, can remain as a flagellate in culture for years (Balamuth, 1956).

Another peculiarly interesting organism of a similar kind is *Histomonas meleagridis* (see Fig. 9.3) which is an amoeboid organism found in the liver of turkeys and responsible for the disease known as Blackhead; but it also exists as a flagellate with a varying number of flagella when living in the caecum of pheasants or domestic fowls (Wenrich, 1943) and under certain conditions of culture (Tyzzer, 1934). The turkeys apparently become infected by swallowing eggs of the nematode worm, *Heterakis*, which have been penetrated by *Histomonas* organisms when in the gut of the domestic fowl. It would be interesting, in view of the present discussion, to know what particular feature of the environment determines this difference of morphology. Another curious feature of this creature is that it has no cyst or other resistant phase. It seems to use the resistant egg of the nematode instead.

THE PROBLEMS OF COLONY FORMATION

These versatile amoebae seem to point the way to further evolutionary advance. Certainly a cell which can be partly amoeboid and partly flagellate either in time or space, or both, would appear to be at a distinct advantage over cells which are immutably either one or the other. Moreover, a colonial form, combining both flagellate and amoeboid activities, would as Metschnikoff showed (1886) have further advantages over the isolated organisms in that, in addition to other possible benefits arising from co-operation between the cells, both activities might go on at the same time. *Volvox* has been cited as an example of the successful colony formed by combining flagellate and photosynthetic activities. The sponge blastula can perhaps be cited as an example of an organism combining flagellate and phagocytic (holozoic) activities.

In the sponge embryo, large posteriorly-situated amoeboid cells, digesting bacteria, may well pass food materials into the body cavity and help to support the motor activities of the anteriorly-placed flagellate cells while these disperse the species by carrying the whole organism away to new feeding grounds. Adequate dispersal is, of course, a necessary quality for successful colonization and many species accomplish this by more or less specialized larval forms. It seems probable, however, for the reasons which have been outlined, that the sponge blastula, although perhaps initially a product of secondary specialization for purposes of dispersal only, approaches more nearly to the primitive colonial metazoan with advantages in other directions also.

The relationship between the two groups of cells may well be mutual, and the flagellate anterior cells may add substances to the fluid in the central cavity which are necessary for the existence of the posterior cells. For example, it has been shown that the amoeboid form of *Naegleria* is adopted in all concentrations of NaCl above M/16; if therefore the anterior cells of the sponge collected NaCl and poured it into the blatocoele cavity in sufficient quantity, then the posterior cells, if they behaved like the amoeba, would be stabilized in the amoeboid form. This is not to suggest that NaCl is the necessary and sufficient condition, although such simple factors are worth bearing in mind, but other metabolites from the anterior cells may influence the activity of cells of like kind and of a different kind in opposite directions. Among the protozoa there are many examples where it has been shown that the by-products poured into the culture medium of an organism will inhibit the activity of like organisms but favour the activity of other types. This sort of relationship between anterior and posterior cells is obviously of great potential importance.

As pointed out earlier, blastulae or early embryonic forms combining more or less motile anterior cells with more or less phagocytic or food-storing cells at the posterior pole, are characteristic of the early life of a great number of Metazoa. Indeed, the exceptions to this generalization are almost certainly specializations brought about in response to some particular environment or peculiarity of development.

In other words, the primitive Metazoon is likely to have been an organism which combined cells of two types, saprobiotic, flagellate cells and holozoic, amoeboid cells in a relationship of symbiotic co-operation. The example of *Naegleria* shows that these two forms of activity are not necessarily impossible to achieve within one and the same cell. In primitive Metazoa it may have been possible, and in the young larvae of existing Metazoa it may still be possible, for one form of cellular activity to give place to the other and for the change to be reversible. Local conditions may determine the nature of the activity of the cells at any one time. This reversibility may even remain as a possibility later in life and may, in some cases, persist into higher forms, though in general, the more specialized a cell becomes the less liable it is to change its characteristics.

A few remarks about the possible origin of the colonial form may not be out of place since, up to this point, it has been rather tacitly assumed that it has arisen from the aggregation of individual organisms, or at least by the failure of the products of cell division to move apart from each other after the division is over. Probably some form of cohesion among the daughter cells is most likely, but the time at which differentiation among the associating units occurs may be very variable. Several views on the mechanism of colony-formation can, of course, be held and there is not the evidence available to say which of them, if any, is most likely to have been the one actually followed. For example, both in the choanocytes of the sponge, and in the individuals of *Naegleria*, it is often observed that flagellar activity may be simultaneously occurring at one pole of the cell, while amoeboid pseudopodia are protruding at the other. Should such a cell divide in such a way as to separate the two functional parts or become binucleate and one nucleus travel to each half and then the cell divide, but with the daughter cells not moving away from each other after their fission and remaining attached by some form of mutual cohesion, then a potentially colonial form would be in the making. The necessities for this theory are (1) spatial separation of activities within the cell, which is seen to occur in some cells, and (2) cohesion on the part of the daughter cells. This does sometimes happen, though, on the whole, daughter cells in most cases tend to pull away rather vigorously from each other and then to separate completely. A theory of the origin of the colonial form somewhat along these lines has been developed by Hadži (1949) in which he derives the primitive Metazoon from the large multinucleate ciliates which may show localization of ciliary activity at one end and more holozoic activity at the other. Furthermore, he correlates the rather elaborate organelles within such ciliates with the organs of the simplest flat-worms. The syncytial development and incomplete separation of cells in the early stages of the formation of the rhabdocoele larvae is, of course, a strong argument in favour of such a hypothesis and there is much to be said in favour of the view which places these organisms near the foot of the metazoan tree. However, these syncytial forms of embryos are rather exceptional as compared with corresponding embryonic stages in which the blastomeres are completely separate from each other, though remaining adherent. Moreover, since some tendency for cells to cohere is, in any case, such a fundamental necessity for the development of colonial forms, more weight should perhaps be given to the study of those protozoa which do show a tendency to produce either colonial forms or cell aggregates: and this brings us back either to such organisms as the Mycetozoa with their extraordinary colony-forming powers under the influence of acrasin (Shaffer, 1956), or to the colonial flagellates and their relatives, the Mastigophora; or, interestingly enough, to *Naegleria gruberi* once more.

When growing on the usual agar slopes the *Naegleria* have a rapid growth phase which lasts for several days; at the end of this time growth becomes slower, and more and more of the amoebae begin to form cysts in which they may remain dormant for long periods. These cysts, quite unlike the amoebae from which they are derived, readily stick together, so that clusters of them form and these remain as clusters even when washed off the surface and suspended in watery media. However, more pertinent perhaps than this stickiness of the inactive cysts is the observation that when a group of amoebae, which are approaching the cyst stage, are washed off their agar slope and replanted on a fresh sterile agar slope, after several washings in sterile distilled water, then the cells quickly aggregate together in a single layer to form an apparently continuous sheet of hexagonally-shaped amoebae, in a manner closely resembling the packing of epithelial cells on the plasma surface in a tissue culture, and there is evidence from the effects of fixation that such cells are actually adherent to each other, very like genuine epithelial cells (Figs. 9.4. and 9.5). It appears from this observation as if the cell, when it is approaching the period of cyst formation, can secrete something on its surface or in some other way modify its surface so that it becomes adhesive to its next-door neighbours, or at least remains in intimate association with them.

In *Naegleria* then, and perhaps in other similar species also, there are to be found the three modes of cell behaviour which provide the minimum requirements for the development of a multicellular organism on lines similar to those found in the embryos of sponges and elsewhere. *Naegleria* can live as amoeba, as flagellate, or in temporary colonial form. The only further requirements therefore seem to be the simultaneous development and co-ordination of all these activities in such a way as to lead to self-supporting colonies of cells displaying, or capable of displaying, both flagellate and amoeboid activity. The simplest free-living (i.e. not sessile) colonial form would then emerge as a structure of flagellated cells not unlike *Volvox*, but with the presence of a number of amoeboid or phagocytic cells providing for the main reserve supplies of energy, instead of the chloroplasts as in *Volvox*, Such a hollow, spherical or roughly spherical colony of mutually adhesive cells would contain within itself an enclosed portion of the environment, and it would only be a matter of time before this region could become exploited and colonized by cells dropping into it from the surface, or perhaps by the whole of one hemisphere pushing into it in order to obliterate it. This shows signs of happening in *Volvox;* for in *Volvox* there are cells which loose their flagella and then sever their contact with their neighbours and thus drop into the cavity, there to set up new *Volvox* individuals either sexually or asexually. In sponge embryos a very similar process has been described by which cells leave the surface layer and enter the blastocoele cavity. These

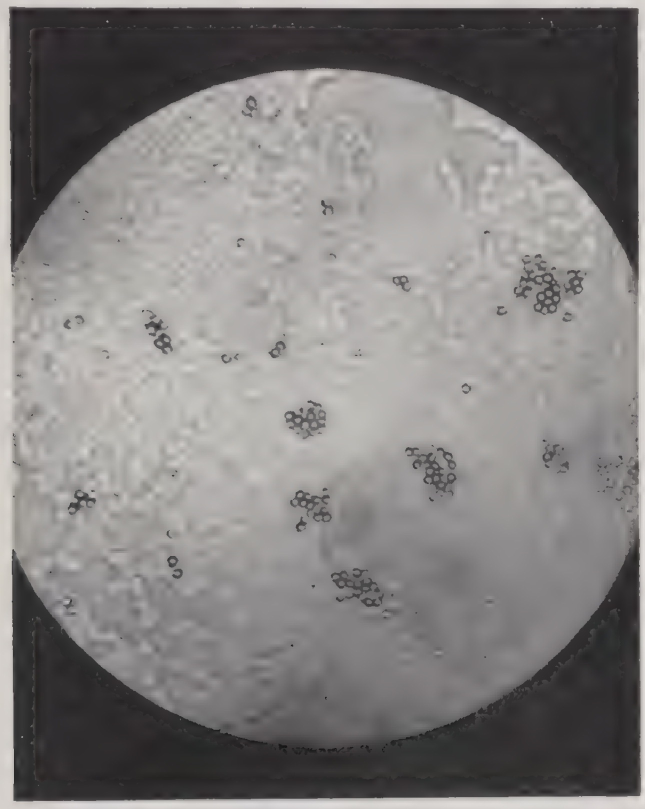

Fig. 9.4.
"Epithelial" growth of *Naegleria gruberi*. Note also groups of cysts.

cells which thus drop in from the two types of surface cells, flagellate cells and amoebocytes, presumably maintain something of their original differences. Indeed, it has been shown that those derived from the flagellated cells lose their flagella and become scleroblasts or, by modification, pore cells and dermal "epithelium", while those from the posterior cells or archaeocytes become the amoeboid cells and the germ cells of the sponge. The flagellate cells thus appear to be potential mechanocytes and epithelial cells and the archaeocytes are the potential amoebocytes.

FIG. 9.5.

"Epithelial" growth of amoebae, under greater magnification than in Fig. 9.4. Each amoeba measures about 14μ in diameter.

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CHAPTER 10

CELL FAMILIES AND THEIR ORIGIN

1. *The primary step in differentiation*

THE suggestion therefore emerges from the study of *Naegleria* and the sponge embryos that, if the concept of a phylogenetically primitive metazoan embryo as being not unlike the existing embryos of the calcareous sponges is justified, the basis for the differentiation into epitheliocyte, mechanocyte and amoebocyte already exists in the behaviour of such organisms as *Naegleria*. If this is so, then the study of this organism and of the conditions which determine its behaviour may be expected to disclose factors relevant to the study of the primary differentiation of metazoan cells.

As remarked earlier, the difference between the amoeboid form and the flagellate form, though certainly striking, is not such that the process, in *Naegleria* at least, cannot be freely reversed. Some intermediate states are also certainly possible. It is not surprising then to find that, in many embryos, the cells are not sharply divisible into the two camps as in the sponges quoted, or, for example, in the ascidian *Ciona*, in which the cells of the postero-ventral pole give a strong peroxidase reaction while the anterior cells give none (Ries, 1937), but rather that there is more often a gradient throughout the organism between the anterior and posterior poles along which there is a tendency to produce flagellate cells anteriorly and amoeboid cells posteriorly. Moreover, the manner in which the cells of the inner mass or mesohyl are formed from the surface cells certainly varies from species to species both in the time and the position of entry of the cells. These differences would clearly be related to the sort of variety that is found in the manner and quality of mesenchyme formation, of mesodermal folding, and in all the other complications of the embryology of the various animal groups. All embryos would have this in common, however, that the primary ingredients are cells showing epithelial behaviour and existing in continuous sheets. From these sheets there must sooner or later be produced some cells, with more or less polarized mechanocyte-like behaviour and others (amoebocytes) showing random unorientated, amoeboid activity; thus among the cells showing epithelial activity two opposite tendencies become evident in their behaviour:

one may be termed mechanoblastic and the other amoeboblastic. In some cases this divergence may occur early and sharply, and account for the presence of two manifestly separate types of epitheliocyte, as in the calcareous sponges. In other cases the divergence may be delayed or masked, and only appear later, so that the early blastula apparently has only one class of adhesive epitheliocytes as in certain echinoderms; even in this case there would probably be some form of axial gradient.

Factors affecting the Amoeboid–Flagellate Transformation

The gradient from anterior to posterior pole in an embryo is subject to modification by external conditions and the activity of either pole can be experimentally accentuated or suppressed. For example, it is well known that the action of lithium ions on sea urchin and other embryos is to suppress the "animal" (anterior) pole cells and to enlarge the "vegetal" pole to such an extent that gastrulation cannot take place normally and an "exogastrula" may be formed. It is therefore of some interest to observe that the addition of lithium salts to the medium which would otherwise favour the conversion of amoeboid forms of *Naegleria* to the flagellate form, suppresses this action over quite a wide range of concentrations. The amoeboid form meanwhile remains normally active. Similarly the absence of SO_4^{--} ions from the medium tends to produce animalization of embryos indicating that SO_4^{--} ions are necessary for the proper action of the vegetal cells (Herbst, 1904; Lindahl and Stordahl, 1937; see also Needham, 1942) and SO_4^{--} ions are found to favour the amoeboid form of *Naegleria.*

Another interesting observation along similar lines is that the addition of choline and of certain quaternary ammonium compounds, e.g. tetra-ethyl-ammonium chloride, pentamethonium, etc. to *Naegleria* in the amoeboid form also prevents the conversion to the flagellate form (Willmer, 1956b). The interest here lies in the fact that the addition of choline and certain quaternary ammonium compounds (e.g. tri-methyl-ethyl ammonium chloride) has been found to increase the numbers of amoebocytes (i.e. macrophages) in tissue cultures from skeletal muscle and other tissues from vertebrates, though it is not yet clear how these increased numbers are produced (Chèvremont and Chèvremont-Comhaire, 1945; Thomas, 1937). They could arise by conversion of the mechanocytes into amoebocytes or by the stimulation of any existing amoebocytes to increased activity and growth. The amoeboid form of *Naegleria* is presumably, if the arguments outlined above are correct, comparable in certain respects with the macrophages or amoebocytes and the flagellate form with the mechanocytes. It is perhaps pertinent to the general theme of the evolution of cell physiology to comment on the fact that these methonium compounds, which are used as rather specific ganglionic-blocking agents in mammals and man, have this strong action on cellular differentiation in *Naegleria.* Some recent work on *Naegleria* (Pearson, 1958) also indicates that certain steroids of importance in the physiology of mammals are involved in the stability of the amoeboid state in *Naegleria.*

Methylene blue, when applied in distilled water to cultures of *Naegleria,* also has a powerful action in preventing amoebae from assuming the flagellate form, which they would otherwise do in distilled water. Moreover, numerous experiments show that the cells in different parts of various embryos have rather specific responses to the addition of methylene blue (Spek, 1934, 1938). However, the physiological actions of this particular substance are liable to be complex; they may depend on its effects on respiration, on ion-transport (Koch, 1954) on its antagonistic action to acetyl-choline (Heymans, 1923; Cook, 1926) on its inhibition of cholinesterase (Augustinsson, 1948), or on the fact that it contains a quaternary ammonium group, though there is some evidence that the last two properties, at least, may be linked. Thus it is not easy to decide which action is being of importance in any one instance.

The action of methylene blue on cholinesterase is one which may not be entirely irrelevant to the present situation; since there is increasing evidence for the importance of cholinesterase in relation to the activity of ciliated and flagellated cells and in relation to ion transport (Kirschner, 1953). Some of the pertinent observations may be briefly summarized. The nerve-free tissue of the gill-plates of the mussel, *Mytilus edulis,* can form acetyl-choline, and such tissue is largely composed of ciliated and flagellate cells. In low concentrations both acetyl-choline and eserine increase the ciliary activity of this tissue but depress it in larger concentrations, as though the formation of acetyl-choline was a necessary part of the activity of the cells (Bülbring, Burn and Shelley, 1953). Similar observations on the action of acetyl-choline have been made on the tracheal epithelium of the frog (Seaman and Houlihan, 1951). The human placenta produces large quantities of acetyl-choline and this has been localized to the fine processes on the cells of the chorionic villi (Chang and Wong, 1933; Wen, Chang and Wong, 1936). Cholinesterase activity rises in sea urchin eggs when they become free-swimming (Augustinsson and Gustafson, 1949), and it is perhaps significant that treatment with lithium salts lowers the cholinesterase activity and "vegetalises" the embryo simultaneously. Cholinesterase has been detected in flagellate trypanosomes (Bülbring, Lourie and Pardoe, 1949) and also in the ciliates *Paramecium* (Bayer and Wense, 1936) (though this has been contradicted by Mitropolitanskaya, 1941) and *Tetrahymena* (Seaman and Houlihan, 1951), but not in the malarial *Plasmodium* (Bülbring *et al*, 1949), an observation which though obviously not significant by itself, yet is consistent with the general pattern which seems to be emerging, namely that the presence of acetyl-choline and cholinesterase are correlated with the activity of ciliated and flagellated cells. On the other hand, there is no good evidence for any action of acetyl-choline on *Naegleria* or for any effects of specifically poisoning cholinesterase by eserine of D.F.P. (diisopropylfluorophosphonate) at the appropriate concentrations. The cholinesterase content rises in amphibian embryos when the neural folds are formed (Boell and Shen, 1944), and these folds show flagellar movement in the frog, which is quickly followed by ciliary movement elsewhere in the epidermis (Assheton, 1896; Twitty, 1928). In this case it is possible that the enzymic activity may correlate more with this feature than with the actual neural activity as has been assumed. Finally, the spermatozoa of the pig have been shown to have a high cholinesterase activity and to be stimulated by acetyl-choline (Sekine, 1951).

All these observations seem to point to the importance of acetyl-choline or cholinesterase in the ciliate or flagellate form of cellular activity. It thus seems possible that choline metabolism, or some form of acetyl metabolism, is characteristic of the anterior cells of embryos and of the their derivatives, the mechanocytes. Furthermore, the evidence suggests that choline may be an end-product of this metabolism and in this connexion the observation that choline increases the numbers of macrophages in tissue cultures may be important when considering the problems of the interrelationships between cells of different types, and of the agents which stimulate cells within the animal body. If, for example, the cells of one group are making and liberating choline or acetyl-choline, and those of another group are stimulated by these agents, then the beginnings of a chemical transmitter-mechanism are already in evidence. There is, however, as already stated, no evidence, so far, that acetyl-choline, in doses comparable with those effective on other systems, has any action on the flagellate form of *Naegleria* even in the presence of eserine, though choline itself in higher concentrations does cause them to change to the amoeboid form. Similarly, it should be emphasized that the evidence that quaternary ammonium ions by themselves can turn mechanocytes into amoebocytes in tissue cultures has not been altogether confirmed.

There are, however, other observations on the behaviour of *Naegleria* which are probably relevant. Experiments with simple chloride solutions show that the presence of different positive ions in the medium has a great effect on the numbers of amoebae which become flagellate. NaCl, KCl, $CaCl_2$, NH_4Cl and particularly $MgCl_2$, all help to maintain the amoebae as such and to suppress their change to the flagellate form (Fig. 10.1). A consideration of the effects of these salts and of the exactly opposite effects of dilute solutions of sodium lactate and bicarbonate which favour the assumption of the flagellate form, indicate the extreme importance of the ionic

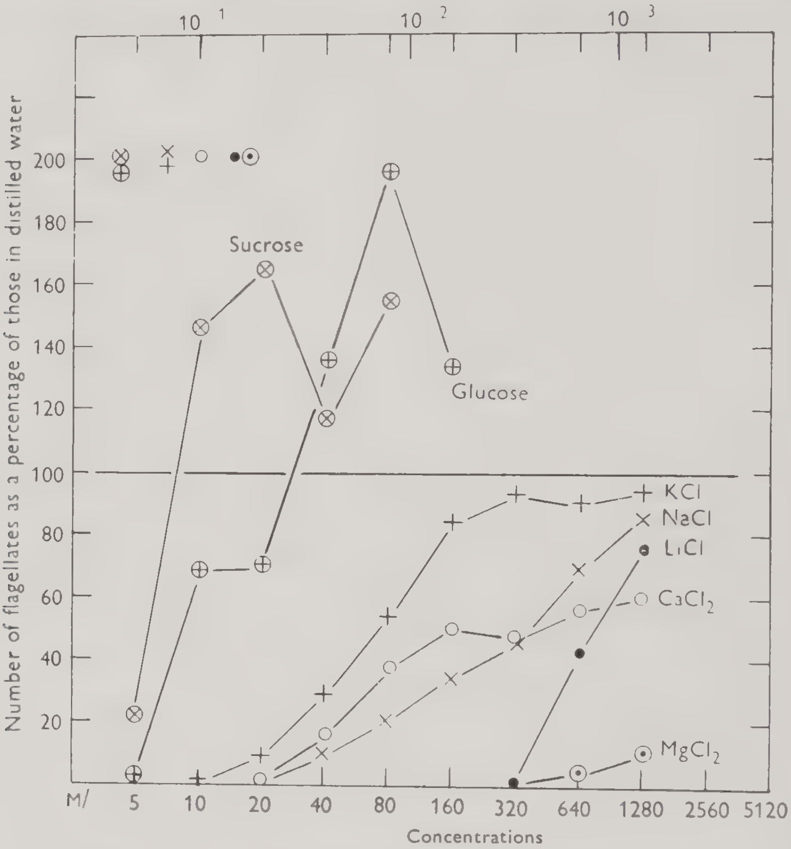


FIG. 10.1

The effects of sucrose, glucose, and certain cations on the behaviour of *Naegleria gruberi*. The curves represent the effects of different molar concentrations (of the chlorides) on the numbers of flagellates which form as compared with the number which form in parallel experiments in distilled water alone (standardized at 100). All the salt solutions used depress the formation of the flagellate form; sucrose and glucose appear to encourage it, but this may simply be because they act as food, so that the amoebae remain in better condition and even multiply. The maximum concentrations at which normal amoebae were observed to be active in the amoeboid form are shown by the appropriate symbols in the top left corner. (Willmer, 1956.)

composition of the medium with respect to the form assumed by the *Naegleria* (Fig. 10.2). In fact, to make a broad simplification, the data so far obtained are consistent with the view that *Naegleria*, in equilibrium, tends to maintain a certain cation concentration within itself.

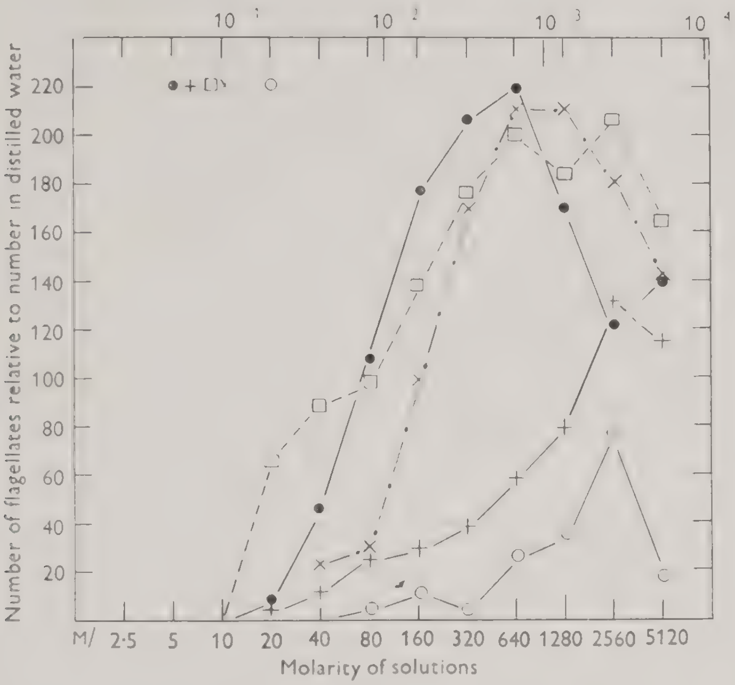


FIG. 10.2

The effects of various anions on the behaviour of *Naegleria gruberi*. The ordinates express the numbers of flagellates formed in the different concentrations of sodium salts relative to the number formed in distilled water, which was standardized at 100. The isolated points (top left) illustrate the concentrations at which amoeboid (as opposed to flagellate) activity is suppressed.

□ - - - □ sodium bicarbonate
● ——— ● sodium lactate
○——— ○ sodium sulphate
× —·— × di-sodium hydrogen phosphate
+ ——— + sodium chloride

The nature of this cation, e.g. Na^+, K^+, Ca^{++} or H^+, for example, is at present undetermined. In *Naegleria* itself, Na^+, K^+, Ca^{++}, and NH_4^+ all behave rather similarly though the concentrations of each in the medium necessary to suppress the flagellate form all differ to some extent. The experiments with lactate and bicarbonate suggest that the cell's internal content of hydrogen ions may be important, and act in a manner opposite to that of other cations, for the acid conditions which probably develop within the amoeba as the result of the action of these ions favour the flagellate form; this is indeed an interesting point because Spek (1934, 1938) has observed by the use of brilliant cresyl blue that in the embryos of certain calcareous sponges the anterior flagellate cells are acid and the granules in the posterior cells are alkaline. However, the situation is obviously not simple, and it does not appear to be universally true that the cytoplasm at the animal pole is acid and that at the vegetal pole alkaline, for the exact opposite is apparently true in the worms *Nereis* and *Chaetopterus*.

If the medium is such that cations tend to be lost from the cells of *Naegleria*, then the flagellate form is assumed; and, alternatively, the amoeboid form becomes the appropriate form when cations tend to be gained rather than lost by the cells.

A somewhat similar state of affairs is seen in the formation of gametes by *Chlamydomonas reinhardi* and *moewusii* (Sager and Granick, 1954; Lewin, 1953). The vegetative cells feed on agar slopes and have no flagella; but, if distilled water is added, then flagella appear and the flagellate forms, of which there are two types, "plus" and "minus", then constitute the gametes, and fusion of the two types may occur. These organisms, which are dependent on photosynthesis, are provided with ammonium nitrate as a source of nitrogen and the addition of this substance to the distilled water delays the onset of mating, and it would be interesting to know whether this substance is acting only as a source of nitrogen or whether the delay in gamete formation is caused by the ammonium ion as such, just as the ammonium ion interferes with flagellum formation in *Naegleria*. Another interesting feature of the flagellum and gamete formation in *Chlamydomonas*, is that it requires the action of light, or at least is hastened by the action of light, and, as will be discussed later, there is the suggestion of a rather intimate connexion between flagella and the carotenoid pigments, some of which are directly concerned with photosensitive processes both in plants and animals.

IONIC BALANCE, CELL FORM AND THE STABILITY OF THE BLASTULA

However the ionic-balancing mechanism may ultimately turn out to be organized, there is already sufficient evidence to suggest that "ionic balance" is a matter of primary significance in relation to the two morphological forms of *Naegleria gruberi* and probably of *Chlamydomonas* also. Indeed, it may well be that the ionic movements across the cell membrane are the important differences and the presence or absence of flagella are secondary manifestations of these differences. Both may, of course, depend on the essentially fibrous or globular state of the proteins concerned in the formation of the cell structures and membrane.

If, therefore, this idea of ionic stability can be legitimately translated to sponge embryos, and perhaps to others also, a whole new vista of problems in relation to the colonial form comes into view. The fluid of the blastocoele cavity of embryos may or may not be the same as that of the outside medium. In general it is probably not the same and in some cases, at least, it has been shown to contain mucoproteins (Monné and Härde, 1950) or other proteinaceous material (Smith, 1958). It is not difficult to visualize, however, that its composition might play a very big part in altering the activity of the cells which are in contact with it, and that its regulation might be an achievement of very great evolutionary importance. This sort of regulation can be well carried out, as demonstrated again and again in various physiological situations by balancing the opposing actions of two types of cells. If the cells surrounding the blastocoele cavity were all of one type, it is easy to visualize that they might, in the ordinary course of their activities, pass an excess of fluid, ions or metabolites into the cavity and these might go on accumulating there to such an extent as to be deleterious to the system as a whole. The presence of cells of an opposing type could theoretically provide the necessary antidote. To oversimplify the present situation for the sake of emphasis, if the amoeboid cell has a surface membrane which actively ejects Na^+, for example, and the flagellate cell one which actively pumps Na^+ inwards into itself, and there is now direct evidence for this sort of activity in *Naegleria* (Willmer, 1958), then the blastocoele cavity, surrounded by a mixture of these two types of cell, could have its Na^+ concentration regulated by the appropriate action of the two groups of cells, without danger of it running away in either direction. Moreover, for the stability of the organism it would be expected that the activity of the cells of the blastula would be determined more by the composition of the internal fluid than by that of the external environment, though of course alterations in the latter, in so far as they affected the former, could not be ignored.

The position is naturally not quite so simple as that just outlined, because two different surfaces are involved in the cells which separate the internal and external fluids. There may be ion and water movement between the cells and their external environment, and also between the cells and the blastocoele cavity; and the cell membranes towards the respective environments may be different. An alternative interpretation, which in the relative darkness illuminated only by the feeble light of the existing evidence is equally tenable, would be that the flagellate cell can, by creating a "current" through itself, accumulate Na^+ from dilute solutions, as in *Naegleria*, and at the same time eliminate excess water, while the amoeboid cell is adapted to strong concentrations of the medium and can eliminate salt against a gradient. It is too early to say exactly how the two groups of cells do actually combine but nevertheless it is easy to see that, without some regulatory mechanism, the composition of the blastocoele fluid might run away progressively in one direction or the other, to the probable detriment of the organism. The same strictures would apply also to the total water content, but blastulae do not normally either swell or collapse except as part of some fully regulated and "natural" process, e.g. as at gastrulation. By way of contrast to this and to see the consequences of an unbalanced system, it is relevant to bear in mind that when tissue cultures of the chick's kidney are made, there is a tendency for the cut tubules to close off and to form small vesicles. When this occurs, those vesicles which have arisen from the proximal convoluted tubules tend, under some conditions, to absorb fluid and to swell up, while those from the distal tubes tend to collapse (Chambers and Kempton, 1933): this is certainly suggestive of fluid movements predominantly orientated in opposite directions by the two kinds of cells.

While this hypothesis, as just outlined, is in all probability much too simple and naïve, and even though it is not yet supported by much direct evidence, the problem raised by the necessity for the ionic and osmotic regulation of early colonial forms must certainly have been important. In the present state of our ignorance the word ionic should be given the widest meaning; exactly which ions (Na^+, K^+, Ca^{++}, Mg^{++}, NH_4^+, or H^+ or even the anions) are the determining agents in any particular case, still remains for further elucidation. Nevertheless, the suggestion of there being a balance between two opposing mechanisms with respect to the main determining agent is at least consistent with general physiological principles.

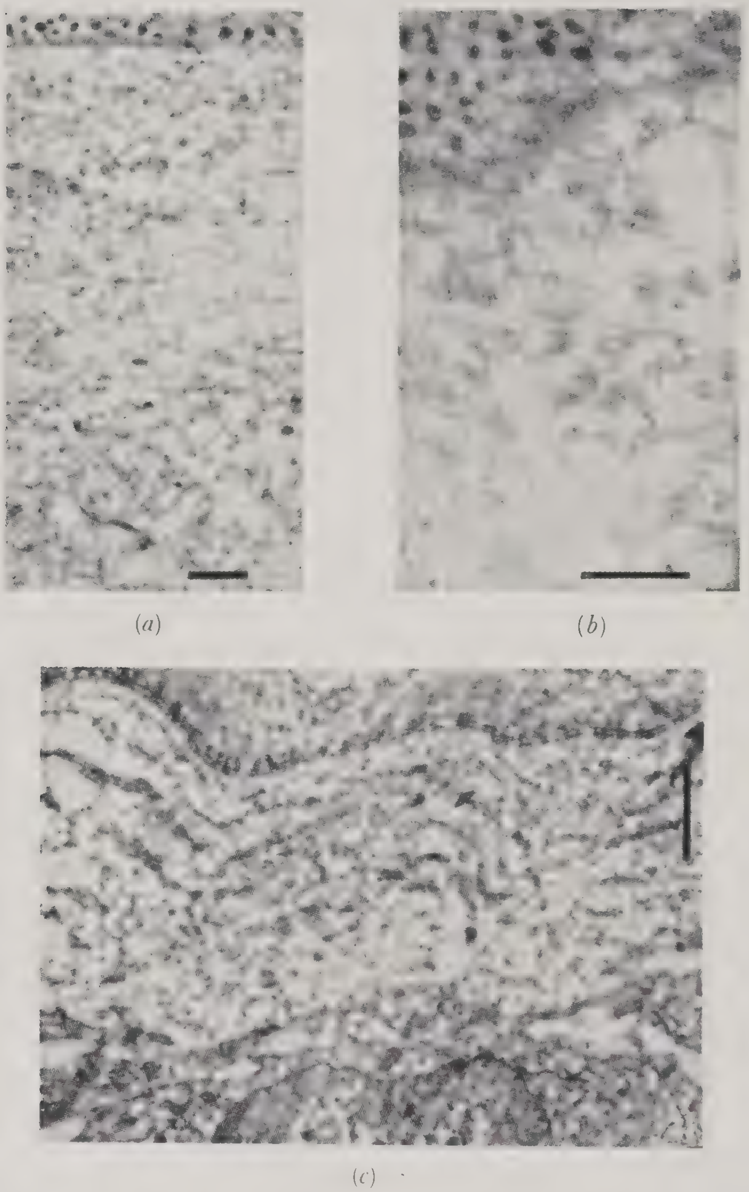
There may thus have been two major factors concerned in giving the advantage to those colonial forms which possess cells of two types. In addition to the provision of two types of feeding mechanism and the possibility of combining one of them with increased locomotion, the regulation of ionic and osmotic equilibrium of the whole organism must certainly be beneficial and is probably a necessity.

Gradients of Activity in Relation to Ionic Balance

An important feature of this kind of hypothesis is that the two opposing groups of cells need not be different in an "all or none way": they could differ quantitatively as well as qualitatively, so that a gradient could exist among the cells, for example between very strongly cation-retaining cells at the anterior (animal) pole, and very strong cation-ejectors at the posterior (vegetal) pole. At some intermediate level the ionic pumping mechanisms might be but little developed in any directional manner within the cells. It is probable that, in general, any given cell would be so set as to work one way or the other, but the extent to which it worked could be quantitatively variable. Some cells, however, in the intermediate zone could well be set at a critical level for reversal of their activity with appropriate changes in the medium.

A very important point in this connexion has been brought out by electron-microscope studies of the surfaces of cells. The early biophysical experiments on cells, and observations on protozoa and other cells isolated in uniform media, tended to suggest that the cell membrane, like an oil-water, or water-air interface, was rather uniform and the idea of a surface which varied from point to point was not generally attractive. Nowadays, however, it is quite clear that at least when they are in situ, cells may show remarkable and localized changes in structure over their surface; microvilli and invaginations are, of course, now commonplace surface phenomena, but the accompanying photograph (Fig. 10.3) of the basal layer of the surface epithelium of an amphibian opens up quite a new vista of localized variations in the surface, and shows not only how the surface may differ with the medium to which it is exposed, e.g. the basal membrane and tissue fluid on the one hand or neighbouring cells on the other, but also how each of these membranes has localized "patches" of entirely different "structure" (Weiss and Ferris, 1958). It must not immediately be assumed, therefore, that an organized cell *in situ* must be "all or none" for any given form of activity, such as ion conservation or ejection; different areas may show different properties. Such an idea of a mosaic cell surface has, of course, been suggested before on histological grounds for epithelial cells (Wislocki, 1951) and also for nerve cells where excitatory and inhibitory effects call for explanation. The mosaic character may always depend to some extent on localized differences in the immediate environment (in this latter case, other nerve terminations) and be of less significance for those cells which find themselves in a fluid medium or which are themselves constantly changing their surroundings, e.g. blood cells and macrophages, etc.

The idea of a gradient of opposing activities between groups of cells is borne out by the behaviour of parts of the amphibian embryo. If the embryo is divided into three regions, an animal portion, a middle portion, and a vegetal portion, and these are then encouraged to develop, it is usual to find that only the middle third will develop normally (Paterson, 1957). Presumably it is only in this region that



(*a*) (*b*)

(*c*)

Fig. 10.3

Electron-microscope photographs of the basal surface of the epithelium of the frog's skin in a repairing wound.

(*a*) The base of the epithelial cell with its "bobbins" is seen at the top: random collagenous fibres below.

(*b*) Tangential section of the base of the epithelial cell, with "bobbins" at the top, then a layer of granules, then a dense precipitate on the outer surface of the basement lamella. Most of the field is filled with collagenous fibrils with some degree of orientation.

(*c*) Section showing the layer of "bobbins" at the base of the epithelial cell, and the orientated collagen fibres of the nearly fully formed basement membrane. (Weiss and Ferris, 1958).

the proper balance between the activities of the more animal and more vegetal cells can be achieved. This gradient of ionic movements would presumably be intimately related to such things as the surface charges on the various cell surfaces and electrical potentials across their membranes, and account for some of the manifestations of the primary (animal → vegetal) gradients which have been detected by staining methods and by other histological techniques. The rather specific staining of the surface of the entomesodermal cells in the embryo of the mollusc *Teredo* (the ship-worm) with silver is probably a case in point and is particularly relevant to this discussion, since it is these cells which first leave the surface of the embryo (Fauré-Fremiet and Mugard, 1948).

The idea of dominance of one region of an embryo over another can now be visualized in a simple way. If, as suggested, cations were pumped into the blastocoele cavity by the anterior flagellate cells, they might, by analogy with *Naegleria*, inhibit other cells from becoming flagellate; suppression of their release, by interference with the activity of the flagellate cells or by their removal, would allow other cells to become flagellate, so that such a mechanism would be consistent with the idea of one part of the embryo dominating over other parts, e.g. the "animal region" dominating over the "vegetal region", and at the same time it would allow reorganization to occur as soon as the dominating region was suppressed or removed.

If these ideas are translated into terms of the cells derived from the primary blastula in higher animals, then the answer to the question of whether, in any one species, there is a hard and fast distinction between mechanocyte and amoebocyte or whether there are intermediate types, must presumably depend on the nature of the "gradient" in the embryo and on the degree of irreversibility of the differentiation into one type of cell or the other. The mechanocyte on these grounds would be regarded as a cell which had such ionic balance, membrane-structure and metabolism that it was habitually orientated to conserve cations, while the amoebocyte and its derivatives would normally be tending to lessen their cation content. The extent to which these activities were developed could be very variable and dependent both on local conditions and on the nature of the cell itself. In the amoebea the change from amoeboid to flagellate form is in the nature of a modulation and the point at issue in the case of mechanocytes and amoebocytes is the question of whether this modulation ever becomes a true differentiation, if we define the latter as being an irreversible change.

In *Naegleria* the behaviour of the one cell can apparently be changed backwards and forwards at will, but the degree to which mechanocytes and amoebocytes in higher vertebrates remain constant and continue to breed true in tissue culture suggests a greater degree of fixation and irreversibility. This ultimate fixation of an initially plastic state is a common embryological and evolutionary phenomenon and is what might be expected. On the other hand, the behaviour of *Naegleria* might be taken to encourage the belief that mechanocytes and amoebocytes in higher animals are not to be regarded as two firmly fixed races of cells with little chance of reversible change from one into the other. Moreover, it must be remembered that a process, which was at one time not only possible but of regular occurrence, might perhaps occur again under the appropriate set of conditions; it would therefore be rash, in view of the evolutionary history of the two cell-types as outlined above, to deny the possibility of the conversion of mechanocyte to amoebocyte and *vice versa*, even in the highest vertebrates. Nevertheless, were such changes to occur they would, as shown in an earlier section, involve radical changes in the whole physiology and metabolism of the cells and not merely changes of shape and visible form. These changes are, however, such as might readily follow from some reorganization of the surface of the cell determined by the ionic content of the cell's environment.

Two Types of Epitheliocytes

With regard to the epithelial surface of the metazoan embryos, there is inherent in all that has been said above, the idea that the surface epithelia of the organism, as they develop, are likely to incorporate more or less of the "flagellate" (mechanoblastic) and "amoeboid" (amoeboblastic) forms of cells according, initially, to their position in the embryo, to the original steepness of the gradient between the two cell types and to the amount of intermingling of the cells that has taken place. It is thus particularly interesting to find that the epidermis of the amphibian neurula is a mosaic of ciliated and non-ciliated cells in varying proportions according to the location on the embryo. (Assheton, 1896)

Where the original gradient in the embryo is steep the differentiation between the cell types is likely to be accentuated, but where the gradient is gradual there may be many intermediate forms of behaviour of cells which could easily be pushed in one direction or the other. In a complicated embryo like that of the chick, the simple primary pattern, as seen in the sponge embryo, is obviously never seen, but there are strong indications of its previous existence and subsequent modification. Grafting experiments, and experiments on the self-differentiation of different regions of the chick embryo in tissue culture, have established the existence of an antero-posterior gradient without much doubt. How this antero-posterior gradient on the more or less flattened blastoderm is to be related to the primary axis of the more primitive spherical embryos is not at present clear, but there is some evidence to suggest that the chick blastoderm can be conveniently considered as a compressed and flattened blastula in which the yolk, in a sense, lies outside the embryo; in this case the blastocoele cavity has become represented by the original split in the blastoderm which divides the epiblast from the hypoblast (Waddington, 1952). If this is so, then the antero-posterior axis of the chick still corresponds more or less accurately with the antero-posterior axis of more typical blastulae: this is an important point and one which receives support from further observations which will be described in another connexion. After this simple and flattened blastoderm has been laid down it is almost immediately complicated by the extensive and elaborate morphogenetic movements which ensue. Although much is known about the general nature of these movements, their immediate causes and effects are often still obscure and there is at present little information concerning anything more detailed than the rather large mass movements of cells which can be followed by vital staining. Nevertheless, the movements which have been described would appear to mix together to a greater or less extent cells which originated in different parts of the embryo, so that by the time the head-folds and neural folds are formed and the somites developed, cells from anterior or posterior parts are probably inextricably mixed, and indeed this mixing in varying proportions may provide a clue to some of the different potentialities of tissues, as will be seen later. In the embryos of nearly all the higher animals the primary pattern is, as in the chick, more or less obscured by the morphogenetic movements of cells. By these movements the cells change their proportions quite extensively as they bring about the folding of the various layers and the sorting out of organ and tissue rudiments.

The argument which is being made here can be summarized as follows. The cells of an embryo are all primarily epitheliocytes, but they are not all identical; the first step in differentiation divides them into two distinct groups of cells. First, there are those which were initially concentrated near the animal pole and correspond to the flagellate cells of the simple blastula and which perhaps among the other properties, are conservers of cations, with respect to their relationships with the external environment. This group of cells gives rise to the mechanocyte type of cell if its members leave the primary epithelium and sink into the mesohyl. The second group of cells, initially concentrated around the vegetal pole and corresponding to the amoeboid cells, are probably concerned with conserving water and eliminating salts to the outside world. These cells, on sinking into the mesohyl, become the amoebocytes. These two groups represent the two extremes of activity, but intermediate cells with these characters less strongly developed must also be presumed to exist. Moreover, among these intermediate forms it may be relatively easy to change a cell over from being, let us say, weakly amoeboid and cation-ejecting to becoming mechanocytic and weakly cation-conserving. The greater the initial distance from what might be called ionic neutrality the more difficult would it presumably become to change the cell from mechanocyte to amoebocyte or *vice versa.*

METABOLISM AND THE PROCESS OF DIFFERENTIATION

Conservation or otherwise of ions has been used here as the criterion for cell differentiation because there is some evidence for its importance, but obviously the cation content of a cell is linked with many other aspects of its activity. The nature and special orientation of its proteins, and whether they are globular or fibrillar, may cause great differences in the ions which are bound to them and thus necessary for the cell. The production of acid or alkaline metabolites must also determine the movement of ions in or out of the cell since only limited changes of internal pH are possible. Thus ion movement—though important in itself—may merely be reflecting the nature of the proteins or other metabolites which are being formed or stored within the cell.

In this connexion the localization of enzymes and metabolic patterns in the cells of developing embryos is clearly of the utmost importance, but the interpretation of such localization is not easy. With reference once more to *Naegleria*, it may be remarked that most of the active feeding seems to go on in the amoeboid phase, so that enzymes concerned with this may then be well developed and become less in evidence when the cells turn flagellate. These systems in this case would seem to be secondary to the primary determination of form which is dictated by the ionic equilibrium. It is, however, important that gradients in numbers of mitochondria (Gustafson and Lenicque, 1952, 1955; Reverberi, 1956) in the distribution of SH groups (Runnström and Kriszat, 1952; Beatty, 1951) of cytochrome oxidase (Reverberi, 1956; Berg, 1956; Weber, 1956), succinic dehydrogenase and other enzymes (Gustafson and Hasselberg, 1951; Ries, 1937, 1939; Weber, 1956), and, of particular interest in connexion with some effects to be discussed in later chapters, in sensitivity to oestradiol

(Runnström and Kriszat, 1954) have been found in many embryos, not to mention the storage of yolk, mucoproteins, fat and other detectable constituents. Oestradiol tends to suppress the endomesodermal regions of the sea-urchin embryo. Finally, in many protozoa, a sexual phase, with the formation of flagellate gametes, often occurs when a feeding phase is coming to an end. The separation and distinction between cause and effect therefore, between metabolism and ion conservation, between the advantages of almost static amoeboid existence or of swimming and dispersal, are not easy to make.

With regard to the localization of mitochondria and of the oxidizing enzymes, there is this to be said. Such structures and systems are concerned with supplying the energy necessary for the activities of cells, and their number and concentration are therefore much more likely to be diagnostic of the work which the cells are being called upon to do than of the type of cell that is doing the work. For example, on the hypothesis that a blastula is composed of two types of cells concerned with regulating the composition of the blastocoele fluid, it is easy to imagine that under some conditions the "animal" cells may have to do more work than the "vegetal" cells, while under other conditions the reverse may be true. The numbers of mitochondria, the detectable amounts of cytochrome oxidase, succinic dehydrogenase, and even the capacity for decolorising methylene blue may thus reflect this aspect of cell behaviour more closely than the nature of the essential differences between animal and vegetal cells. In the search for diagnostic characters, therefore, the oxidizing systems alone are not likely to be a very good guide.

It is very tempting to draw parallels between the behaviour of the chick epidermis under the action of Vitamin A, the behaviour of *Naegleria* under different cultural conditions, and the behaviour of the two types of cell in early embryos. *Naegleria* can, under at least one set of conditions, form an apparently uniform population of closely adherent cells almost epithelial-like in character. Under more favourable conditions these may separate into free-living amoebae and these may become free-swimming flagellates. The chick skin consists normally of an apparently uniform population of closely adherent cells with a tendency to keratinize. These cells under the influence of large amounts of vitamin A, produce two types of cells, one essentially a mucin-secreting cell, and the other a ciliated cell. In the case of the mucin-producing cell, autoradiographs show that this type of cell, in contrast to the squamous epithelial cell which requires organic sulphur, can pick up inorganic sulphur rapidly from the medium (Fell, Mellanby and Pelc, 1954; see also Jennings and Florey, 1956) though it may not be able to incorporate it directly into the mucoprotein, and this points to the possible parallel between this type of cell and the vegetal cells of certain embryos, for these have been shown to depend upon the presence of SO_4 ions in the medium while the cells of the animal pole are unaffected by their absence: in consequence embryos are "animalized" by lack of SO_4 ions in the medium. SO_4 ions in the media of *Naegleria* cultures favour the assumption of the amoeboid form. This action of sulphate ions is, of course, not simple and may involve not only ionic equilibrium but the ability of the cell to carry out certain detoxications, to form necessary polysaccharides, or to maintain its calcium balance, any of which could have very far-reaching effects.

Cell Multiplication and the Establishment and Maintenance of Races of Cells

Considerations of this sort foster the belief that much light may be thrown on the differentiation and metabolism of the cells of higher animals by taking into account the source of such cells in both ontogeny and phylogeny. Quite early in the history of animals the cells of the organism must have started to specialize. Cell A became different from cell B, and if that change, which must be assumed to have benefited the organism, perhaps by stabilizing the ionic balance, was a heritable change either by nuclear or some form of cytoplasmic inheritance, then it must also be assumed that future generations would also continue to produce cells A and B. In other words, two families of cells must inevitably result; the nature of the heritable change as it affects somatic cells is, as already pointed out, still problematical. There are numerous observations which show that in many embryos the nuclear material can be interchanged from cell to cell in the early stages without any untoward effects, and that all the nuclei of the early blastulae are equivalent. This, however, does not seem to remain true indefinitely (i.e. in *Rana pipiens* not beyond the gastrula stage) for when the nuclei from endoderm cells in the neurula stage of this frog embryo are grafted into other enucleated eggs differences in character are observed in the abnormal embryos which subsequently develop. Moreover, when nuclei from these abnormal embryos are grafted into other enucleated eggs the characteristic abnormalities tend to persist in this "next generation" (King and Briggs, 1956). Thus there is now some direct evidence of a functional nuclear differentiation among somatic cells. In tissue cultures, cells certainly do breed true for long periods and perhaps, in some cases indefinitely. Moreover, the nuclear form in different adult tissues of the body is generally among the most characteristic histological features, and probably indicates certain differences either in the deoxyribosenucleoproteins themselves or in their relationship with the immediate substrate upon which they work. (See Chap. 7).

Each type of cell certainly has its own distinctive nuclear pattern, and however the chromosomes may follow an inexorable law during mitosis, the resting nuclei of each group of tissue cells nevertheless differ from those belonging to other groups. There is perhaps an interesting pointer in the observations by Barr and his colleagues (Barr, Bertram and Lindsay, 1950) first on the nuclei of nerve cells and later on those of many other types of cell in which the presence of a particular mass of chromatin is correlated with the sex of the animal. This chromatin is thus possibly concerned with regulating the cell in relation to circulating sex hormones, gonadotrophins and the like, and those cells which do not have this chromatin are presumably differently integrated into the sex mechanism. A curious feature of this chromatin is that it has not so far been observed in the cells of the liver or kidney. Further investigation may show that the cell families under discussion (e.g. mechanocytes, etc.) have similar visible modifications of nuclear design, but, so far, the differences between the nuclei of different classes of cells are too nebulous to be of much assistance. In any case the differences between different groups of cells within one organism are much more likely to depend on the action of a limited number of genes than on whole chromosomes, so that visible differences at mitosis may be very unlikely. Amoebocytes in culture, on the whole, tend to have one large nucleolus while mechanocytes under the same conditions generally have two or more smaller ones but this difference is not sufficiently constant to be of much significance, and it is often lost when the cells differentiate or modulate further. However, it may suggest differences in rate of synthesis of protoplasmic materials, and the rate of synthesis of protein could be very important in the transport of ions. When growing *in vitro* the cells in the two groups, e.g. "fibroblasts" (mechanocytes) and macrophages (amoebocytes) have different nucleo-plasmic ratios as measured by visible dimensions (Strangeways, 1929); but again this can hardly be used as a diagnostic feature for individual cells since the results are only statistically valid, and the difficulties of measuring the volumes accurately are very great.

While it is fairly clear that if there are two races of cells A and B, the daughter cells of A are different from those of B, and most probably remain so, it is not yet clear by what mechanism the A cells breed true, and continue to do so till they subdivide again into further subsidiary types A_1 and A_2; nor is it clear how the B cells propagate their kind till they again produce one or more heritable variations, B_1, B_2, etc. The nature of these mechanisms is a problem for the future; but if the various races do breed true, then since B_1, B_2, etc., can only come from B cells and A_1 and A_2 only from A cells, the B family must for ever remain distinct from the A family, unless some very radical reversal of the process of determination occurs. This reversal can undoubtedly occur in the primitive condition, as seen in *Naegleria*, but it is likely that in higher types the capacity for reversibility decreases for one reason or another, very much in the same way as most processes of differentiation become less reversible as evolution proceeds. Recent work has tended to show that the process of differentiation may not be of such sudden onset and immediate irreversibility as at one time supposed, and that the differentiation of cells may depend for some time upon the local conditions continuing to exert their effects on these cells. In other words, fairly large pieces of tissue may be "self-differentiating" from quite an early stage because of the relative stability of of the local environments of their constituent cells, but the individual cells therein may remain labile for much longer if extracted from their normal situation, and they may have to achieve considerable structural changes before irreversibility sets in.

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CHAPTER II

CELL FAMILIES AND THEIR ORIGIN

2. *Cells of the inner mass and the nature of "ground substances"*

IT IS perhaps now appropriate to consider how far the picture of the various main cell types and of their possible mode of origin which has been drawn in the preceding pages can be fitted into the plan of cellular development and differentiation as these processes occur in the embryonic stages of a typical vertebrate. At first sight it may seem to be a very far cry from the embryo of the calcareous sponge to the human baby, and in many ways, of course, it is. Nevertheless, man has evolved and not been created. Foundations laid millions of years ago are still, and will ever be, the foundations for human existence, and it is our problem to unearth these foundations.

As already emphasized almost *ad nauseam*, the separation of tissues into ectoderm, mesoderm and endoderm, while fairly satisfactory as a purely descriptive account of the development of form, is far from satisfactory as a working guide to the development of cellular function. It is therefore necessary to consider if any other pattern can be found which will lead to more trustworthy results from the physiological aspect.

If the implications of the notion that the early blastomeres of the embryo may be rather sharply divisible into two classes are followed up, it becomes clear that, at an early stage, two main types of epitheliocytes must be recognized. It will be remembered that the primary cells of the embryo have all been classed as epitheliocytes at this stage since, in the blastula, all the cells are at first mutually adherent surface-covering cells, thus exhibiting the essential epitheliocyte characters, and no cells have as yet left the surface. There may also be some waverers between these two main types because the differentiation probably follows a gradient in which the most anterior cells are the most firmly of one type and the most posterior cells are most firmly of the other. At first the differentiation may be, strictly speaking, a "modulation" and be easily reversible but, once a true differentiation of this kind has occurred, then the future of these cells has already been determined to some extent and they will normally continue as two separate and well-defined races, from each of which further lines of differentiation may emerge. In the light of experiments on regeneration and more particularly with single cells in tissue culture, some care is necessary in the use of such terms as "modulation" and "differentiation". The distinction between the two processes largely rests on the irreversibility of the latter process. The differentiation of cell groups may certainly be normally irreversible. Tissues become "determined" and then differentiate along definite lines. Work with isolated cells, in tissue culture, however, indicates a much larger degree of plasticity and adaptability than had been previously supposed on the part of the individual cells derived from tissues which are highly differentiated and even adult. Nevertheless, it has been amply shown in earlier pages that mechanocytes, amoebocytes and epitheliocytes are groups which can be separated on grounds of very firmly ingrained properties, even if they are not completely irreversibly separated.

When, later in development, morphogenetic movements occur, these may cause considerable mingling of the cells of the embryo and a shuffling of their relative positions, but at least after a certain stage, i.e. the stage of true differentiation, they are unlikely to modify the primary distinction by reversing that determination of the cells which has already occurred in the very early embryo. Indeed, the stage of true differentiation, i.e. when the cells begin to take on their characteristic function or at least to acquire the structures necessary for this function, may well be the stage at which the cell behaviour as potential mechanocyte or amoebocyte is determined and fixed. Up to that time, potentialities in either direction may be strong or weak according to the position of the cell in the organism, but the cell may still remain sufficiently plastic for fundamental reorganization to occur under altered conditions.

In the ordinary course of events, therefore, when individual cells or groups of cells leave the primary surface of the embryo to enter the mesohyl or inner mass, these cells and groups of cells may include representatives from either or both of the primary sources. In the simplest embryos, those cells which originated in the anterior region from the more polarized cells can be classed together as the mechanocytes, while those originating from the posterior region are the less orientated, randomly amoeboid and probably more nearly totipotent cells, i.e. the amoebocytes. In addition, there may be some less determined cells, derived from an intermediate zone, which maintain the capacity to develop into the one or the other, i.e. they may remain with characteristics belonging to both, and, under altered environmental conditions, are more easily capable of turning one way or the other than the cells nearer the poles. These intermediate cells are probably always functioning, on balance, either as the one type or the other but they are rather easily changed over. If, for example, the basis of the initial differentiation lies in the nature of the cell surface, it is unlikely that the cell membrane orientated for one form of activity is also suitable for the other form, although the possibility that there may be a mosaic surface should not be ruled out. The position may be made clearer by supposing that a strong gradient exists in one direction across the surface of the cells at the animal pole, and an equally strong gradient in the opposite direction exists across the cells of the vegetal pole, then these gradients must weaken and reverse somewhere in the equatorial region, but the exact latitude of the reversal may be variable and dictated in part by external conditions. The cells in the equatorial region therefore may be capable of swinging rather easily from slightly "positive" to slightly "negative" and *vice versa* in response to the local environment. Moreover, it cannot be overemphasized that, when cells are located in an epithelial surface, the opposite poles of each cell may be forced into a different form of activity and this may involve the polarity of the whole internal architecture of the cell. Unlike the free-living *Naegleria* the cells in an epithelium may be faced with a flagellate-producing environment on one surface and an amoeba-producing environment on the other.

When the mechanocytes develop further, they produce the cells of the skeleton, the fibroblasts, the chondroblasts and the osteoblasts, etc., i.e. those cells which have already been grouped together as myxoblasts (see p. 51). They also, by a further or alternative differentiation, produce a second group of cells which include the endothelial cells and myoblasts. On the other hand, when the amoebocytes differentiate they give rise to the blood cells (erythrocytes and leucocytes), monocytes, macrophages, and probably those endothelia which display phagocytic activity, e.g. Kupffer cells of the liver, littoral cells of the bone marrow, etc. The gulf between the two extreme classes (myxoblasts and macrophages) may be to some extent bridged by the myoblasts and endothelia, i.e. it is among these groups that cells of somewhat intermediate type, with a certain number of features in common, are to be found. Moreover, these are the classes of cells which mostly originate embryologically from cells in an intermediate position. In the more simple embryos they arise from the cells which lie between the ectoderm-forming and the endoderm-forming masses. For example, in worms, molluscs and ascidians, the muscle-forming cells are clearly marked out by shape, size, position and particularly by visible cellular inclusions at quite an early stage of development, and are then seen to occupy an intermediate zone between the outer surface and the invaginating endoderm (see Fig. 8.1). Corroboration of this primary differentiation by virtue of position, even in higher forms, comes from some classical observations on the behaviour of the blastoderm of the chick when it is cultured *in vitro* in such a way as to encourage the formation of blood. When tissue cultures are made from different regions of the early blastoderm, there is a general tendency for "blood islands" to develop and these then produce blood cells (Murray, 1932). Blood cells, it will be remembered, are essentially amoebocytes, so that these studies are really investigating the occurrence of amoebocytes in the blastoderm. The blastoderm of the chick can be regarded as a flattened blastula in which the primitive streak corresponds to the blastopore and the anterior end is equivalent to the animal pole. Further examination reveals the fact that the potentiality to produce blood cells is restricted to the posterior three-quarters of the blastoderm being maximal in the region of the primitive streak from which the potentiality falls away in all directions but most rapidly towards the anterior regions. Indeed, the most anterior regions are apparently incapable of producing blood cells, and these are the regions which correspond in many ways to the animal pole of the simpler embryos. Presumably, therefore, these observations are consistent with the view that amoebocyte development is characteristic of the more posterior regions of the chick embryo, just as it is in embryos of more simple pattern. The blood cells, which are essentially amoebocytes, therefore arise from the same region of the embryo in the chick as they do in other species.

The production of mechanocytes or of amoebocytes may be considered as resulting from the primary differentiation of the cells of the early embryo into the two main groups of epitheliocytes; it occurs at the time when certain epitheliocytes, for some reason which is not as yet understood, break contact with their neighbours and leave the primary surface. Once isolated in this manner and perhaps because they now lie in a new environment, i.e. the blastocoele cavity, the cells take on new characteristics and differentiate further. By so doing they again alter their own local environment, and probably also that of cells in their neighbourhood, including of course the layer of epithelial cells which they recently deserted. Very quickly after this, the whole embryo becomes in effect a three-dimensional mosaic so that cells in different regions all tend to acquire new local environments, each of which differs from every other, so that further differentiations and adaptations of the cells continuously ensue.

The Raw Materials for Connective Tissue

Some further study may perhaps be made at this point of the nature and distinguishing features of mechanocytes, because these cells presumably owe at least some of their peculiarities to their previous history. Among the outstanding properties associated with the mechanocytes their propensity to produce an intercellular matrix or "ground substance" composed of both mucoprotein and fibres, has been mentioned. The first visible fibres are argyrophil (i.e. "reticulin" fibres) and these then may become collagenous. The electron-microscope, however, shows that isolated rod-like "tropocollagen" units are actually the first formed elements to be produced; and from these the collagen fibres are built (Gross, 1956). The formation of tropocollagen probably involves the liberation into the surroundings of the cell of such substances as glycine, proline and hydroxyproline, or of substances easily convertible into these (Jackson and Smith, 1957), for these are the essential constituents of the collagen "backbone". In this connexion, glutamic acid is also a substance which is of some interest because it enters into the composition of collagen, and also because it may be a precursor in the formation of hydroxyproline. There is, however, evidence against this idea since C^{14}-L-glutamic acid was found not to be incorporated into hydroxyproline by osteoblasts in tissue culture (Smith and Jackson, 1957); this, however, was based on measurements of the contents of the cells and does not necessarily mean that the process could not go on externally to the cell. Moreover, it has been found in several studies of amino-acid metabolism in tissue cultures that glutamic acid is liberated into the medium during the growth of fibroblasts or mechanocytes (Morgan and Morton, 1957; Lucy and Rinaldini, 1959) and so also is hydroxyproline (Hulliger, James and Allgower, 1957). Tissue cultures of bone fragments do not liberate glutamic acid (Biggers, *et al*, 1958): this may mean that osteoblasts differ from other fibroblasts and do not produce it faster than they use it, or it may simply be that in these cultures, which were organ cultures, it was immediately incorporated into structural fibres and so escaped analysis in the "medium". Glutamine has in some cases been shown to be an essential amino-acid, but glutamic acid appears as a by-product from both heart fibroblasts and myoblasts at least, and neither it nor proline will readily substitute for glutamine in the nutrition of fibroblasts (Eagle, Oyama, Levy, Horton and Fleischman, 1956). Glutamic acid, proline and hydroxyproline, as well as being among those which were inessential (see p. 47) for the growth of strain L-fibroblasts, are also the amino acids which have been found to be actually inhibitory to the growth of heart fibroblasts (Morgan and Morton, 1957), a phenomenon which would be readily understandable if normal growth involves their liberation.

In plant tissue cultures, of carrot tissues, hydroxyproline accumulates in the proteins which are formed during active unorganized cell proliferation. It appears to arise from proline, and the addition of hydroxyproline (but not of proline) to the medium stops the growth (Steward and Pollard, 1958). In fact, it appears as if endogenous proline has to be removed, by incorporation as hydroxyproline in a structural protein, in order that growth shall occur. The comparison with the incorporation of hydroxyproline into collagen fibres in rapidly growing mechanocyte colonies is immediately obvious, especially in view of the fact that collagen production is increased by the growth-stimulating embryo extract (Gerarde and Jones, 1953).

It may also be pertinent to notice that among micro-organisms there is at least one (*Bacillus anthracis*) which has a capsule composed of a polypeptide formed entirely of D-glutamic acid (Kent and Whitehouse, 1955), and this suggests that glutamic acid may be a substance formed in appreciable quantities by these organisms, so that the mechanocyte may merely be exhibiting a character which had its origin in some much more primitive form of life.

Among the primary constituents of the mucopolysaccharides and mucoproteins which constitute the "ground substance" are the amino-sugars, particularly the hexosamines (e.g. glucosamine, galactosamine, etc.) and the corresponding uronic acids which are aggregated and polymerized in a variety of ways, and are often sulphated. Presumably, therefore, these substances are liberated into the medium by mechanocytes in general, and by myxoblasts in particular, and there are certain observations on the fixation of sulphate by regenerating tissue, and by tissues *in vitro* under the influence of cortisone, which could be interpreted to mean that mucopolysaccharide liberation is a property associated with mechanocytes, and with myxoblasts more than with myoblasts (Layton, 1950, 1951). For this reason, therefore, it is perhaps interesting to note that D-glucosamine has been found to inhibit the growth of sarcomata (Quastel and Cantero, 1953; Rubin, Springer and Hogue, 1954) (sarcoma cells have many of the features of mechanocytes), of chick heart fibroblasts (Ely, Tull and Schanen, 1953), and also that of a skin carcinoma (Fjelde, Sorkin and Rhodes, 1956). Moreover, an increase in serum mucoproteins occurs in animals in which sarcomata (S66) are actively growing (Baldwin and Harries, 1958; Shetler, Erwin and Everett, 1950). In inflammatory changes, in the peritoneum, caused by the injection of silica, positive staining with Alcian Blue and metachromatic staining with toluidin blue were found to occur wherever fibroblasts were actively proliferating (Heppleston and Keyser, 1957) and both these are signs of mucoprotein production. Thus, although these observations have generally been made in situations and conditions in which there are many complicating factors, there are fairly clear indications that glucosamine and allied substances are important metabolites of mechanocytes.

Once again, the capsules of many micro-organisms and the cementing substances of certain epithelial cells often contain complexes of amino-sugars, uronic acids and hexoses (e.g. chitins). Thus the extracellular formation of these substances seems to be a very widespread feature of cellular activity.

At first sight the production of glutamic acid and that of polysaccharides and amino-sugars may appear to be entirely unconnected with each other, but there is evidence from yeasts that there is an enzyme under whose action glutamine may directly assist in the formation of amino-sugars from hexoses by providing the NH_3. The reaction is essentially glutamine + glucose → glucosamine + glutamate, though it may be necessary that the hexoses are first phosphorylated (Leloir and Cardini, 1953). A similar situation has been found also in *Streptococci*, for these organisms freely produce hyaluronate in the presence either of glutamine or of ammonia and glutamic acid (Lowther and Rogers, 1953, 1955). Glucose is the precursor and it is possible that uridine nucleotides may be involved (Dorfman and Cifonelli, 1958).

All these facts and observations suggest that, in cellular metabolism, amino-sugars, uronic acids and glutamic acid are very frequently, if not always, the by-product of some basic and necessary metabolic processes. Cells of various types have subsequently dealt with these by-products in their own different ways, storing them, excreting them or utilizing them for creating capsules, cementing substances, intercellular matrices and fibres. In other words, the "ground-substance" of connective tissues, the chitin of arthropod exoskeletons and suchlike matrices should really be regarded as materials obtained by utilizing what would otherwise be the waste products of certain activities essential for some types of cell.

THE SOURCE OF THE RAW MATERIALS

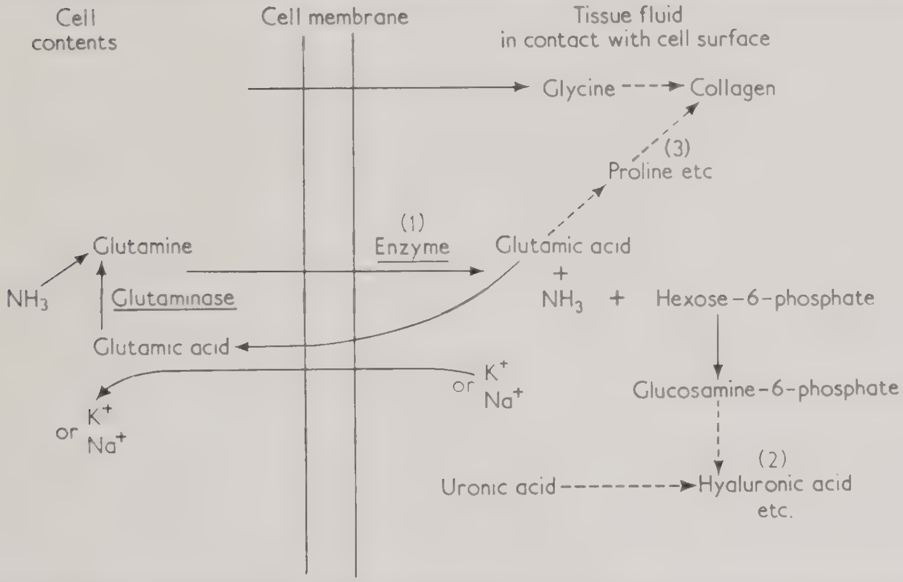
This side of the medal is fairly clear; waste products are turned to advantage. The other side, however, has not yet been deciphered. When it is, it should establish the reasons why these particular waste products are so frequently liberated from cells, by disclosing the nature of the basic metabolic processes which lead to their formation.

It has from time to time been suggested that mucopolysaccharides and mucoproteins are concerned with mechanisms of ion and water transport or equilibrium and their binding power for K^+ is sometimes very different from that for Na^+ (Kulonen, 1952); this is, of course, a point of particular importance in view of the cellular and extracellular distribution of these ions. As has been stressed in the preceding chapters, this association between polysaccharide production and ionic balance would certainly fit many of the facts, and at the same time, evidence is accumulating for the intervention of a "glutamate" system in the same connexion. For example, tissue-slices from brain lose their potassium to a saline medium, but this loss can be prevented by the addition of L-glutamate and glucose to the medium (Krebs and Eggleston, 1949). Similarly, in experiments with excised retinae, glutamate and potassium are found to be taken up by the tissue at approximately equivalent rates (Terner, Eggleston and Krebs, 1950). While these experiments with complex tissues in simple saline media are probably not very reliable or easy to interpret since, for one thing, cells are very readily damaged by washing in such media, the ionic movements can presumably be considered as an attempt to re-establish equilibrium and to prevent further losses and gains by the cells. In these experiments it should be emphasized that glutamate is being used by the tissues and not being produced by them. *Naegleria* assumes the flagellate form in distilled water, but the addition of sodium-L-glutamate to the cells when they are in the flagellate form hastens their return to the amoeboid form (Willmer, 1958). The equivalent amount of sodium chloride is much less quickly effective, though it may achieve the same result in the end. Glutamine is only effective in the presence of NaCl. *Streptococcus faecalis* and *Staphylococcus aureus* both accumulate glutamic acid, with associated glucose utilization, and it is interesting to note that this accumulation is also linked with a rather specific uptake of K^+ (Davies, Folkes, Gale and Bigger, 1953).

These straws blowing in the wind therefore indicate that it might be worth while to investigate the glutamate metabolism and the hexosamine and polysaccharide metabolism of tissues under strictly controlled conditions, not so much from the angle of their part in the production of matrices, etc., but rather from the point of view of why cells require or liberate the relevant molecules. The necessary conditions could be obtained with certain forms of tissue culture or even with such organisms as *Naegleria*.

Some sort of tentative scheme like that outlined in Table 11.1 might suggest a working hypothesis for the manner in which mechanocytes deal with these substances. The essentials of the scheme are that glutamine is converted into glutamic acid and ammonia because the ammonia is immediately enzymically linked with glucose (Leloir and Cardini, 1953). The glutamic acid could then diffuse back into the cell, but it could only do so by carrying with it a positive ion; alternatively, it could be converted to the more neutral state by being converted to proline. The scheme would be expected to be blocked by increasing the concentration of glucosamine or ammonia, for these would tend to block the glutamic acid production, and under some conditions proline and hydroxyproline might act similarly.

TABLE 11.1



(1). As extracted from yeast, (Leloir & Cardini, 1953)

(2). These bind Na$^+$ proportionately more than K$^+$ (Kulonen, 1952) which may be a factor in selectivity of K$^+$ transfer.

(3.) The conversion of glutamic acid to proline is one from an acidic amino-acid to a neutral amino-acid.

A mechanism such as the one just outlined could therefore be at the root of selective ion-permeability of cells, of the formation of ground substances and intracellular cements, and of the production of collagenous materials. Many variations could obviously be played on the theme by cells of different kinds according to their needs.

Directional Movement of Substances across the Cell Membrane

It has been emphasized in earlier chapters that mucopolysaccharides and mucoproteins are sometimes mainly extracellular and sometimes mainly intracellular. Among epithelial tissues there are many in which "mucous" cells, i.e. cells which store mucin, generally in discrete droplets in their cytoplasm, are interspersed with "serous" cells, with ciliated cells or with some other columnar or secretory cells which do not normally store any mucoproteins, though they may liberate them in a less easily detectable manner into their surroundings (e.g. striated borders of duodenal epithelium and of tubular cells of the kidney (Moog and Wengler, 1952)) and, therefore, by way of contrast, it is worthy of comment that some of the yolk granules in sea-urchin embryos are also suspected of containing amino-sugars (Monné and Slautterback, 1950). The ciliated cells of the early blastulae of echinoderms have been shown to secrete mucopolysaccharides into the blastocoele cavity (Monné and Härde, 1950). It could be suggested therefore that cells, from this point of view at least, fall into two broad classes, i.e. mucopolysaccharide-ejecting cells and mucopolysaccharide-storing cells, where the term "mucopolysaccharide" is used very loosely and may in some cases imply little more than hexosamine containing substances or even simpler carbohydrates, though the part played by any necessary protein to which they may become attached cannot be ignored, and protein is certainly present in many blastocoele cavities. This division into mucopolysaccharide-storing and mucopolysaccharide-ejecting cells would presumably be the inevitable result if the production of mucopolysaccharide or its precursors were part of an ion-transport mechanism, i.e. if the passage of Na^+, K^+, or even H^+, for example, through the cell membrane involved something like the formation of glucosamine on the opposite side. Cells needing to collect cations, i.e. for example, those synthesizing acid proteins, would be glucosamine ejectors and those which needed to keep Na^+ or other cation out would automatically tend to accumulate glucosamine, and this they would presumably tend to store, build into mucoproteins or liberate in some indirect manner. The mucoproteins could, of course, also be liberated as such, as for example from goblet cells and other mucous-secreting cells which, incidentally, are cells which yield very little ash upon micro-incineration (Scott, 1933) and thus probably have a rather low inorganic content. Since this could be interpreted to mean that they store mucin faster when they are made to work harder in order to keep the ions out, it is interesting to note that increasing the electrolyte content of the rat intestine increases the activity of the goblet cells in its epithelium (Florey, 1955). Glucosamine has been chosen here mainly by way of illustration; other amino-sugars or their derivatives, e.g. sialic acid and N-acetyl neuraminic acid, could perhaps be used in a similar manner. Indeed, these substances are of particular interest in view of their possible relationship with pyrroles and thus with haem formation. Porphyrin compounds are frequently associated with mucous-producing cells on the one hand and with amoebocyte cells on the other. Thus if the inward movement of amino-sugars and of glycine, hydroxyproline, etc. is characteristic of amoeboblasts, as their outward movement is characteristic of mechanoblasts, porphyrin formation within the amoebocyte may be one of the consequences. However, the more recent work on porphyrin synthesis indicates that this substance is formed from glycine and succinate or α-ketoglutarate, through amino levulinic acid, rather than from mucosubstances (see Wolstenholme and Millar, 1955). The uronic acids also are probably involved in the ion-transporting mechanism since many cells produce polysaccharides containing no nitrogen, and such substances as hyaluronic acid, which occur so frequently as constituents of "ground substances", are compounds of glucosamine and uronic acid. These substances not only have a great capacity for binding water, but they may also be of particular importance because they are among the very few substances which show selective affinity for Na^+, rather than K^+, and might therefore be a factor in determining which cation a cell picked up (see Table 1) (Kulonen, 1952).

The capsules of bacteria often involve the use of amino-sugars which can perhaps be interpreted to mean that the production of these substances is almost basic to living systems and is thus of very fundamental significance. Bacterial capsules fall into several classes according to their chemical constitution; of those containing polysaccharides some consist of acetyl-amino sugars and galacturonic acid; others of glucuronic acid and glucose. It would be interesting to know how the ionic relationships between these bacteria and their respective surroundings differ, if at all.

The idea of cell membranes working in opposite directions i.e. "pumping" inwards or outwards in order to maintain ionic balance in the cell, and of polysaccharides being involved in these exchanges, receives some support from the observations of Dorfman (1958) who points out that mesodermal acid mucopolysaccharides, which are mostly extracellular, contain uronic acids, whereas most epithelial mucins, which are intracellular, do not. The main exception to this is the mucoitin sulphate which occurs in gastric juice (Komarov and Webster, 1935). The suggestion could be made that the significant difference in the distribution of these compounds is not between epithelial and mesodermal tissues but between "intracellular" and "extracellular" products. As already pointed out, the main polysaccharides of the connective tissues are extracellular in origin or at least accumulate outside the cell. Most epithelial polysaccharides so far investigated are those which are temporarily stored in recognizable form within mucous cells, and then liberated. However, this may be too naïve an interpretation. For example, heparin which is probably an intracellular product in mast cells, contains uronic acid. In the

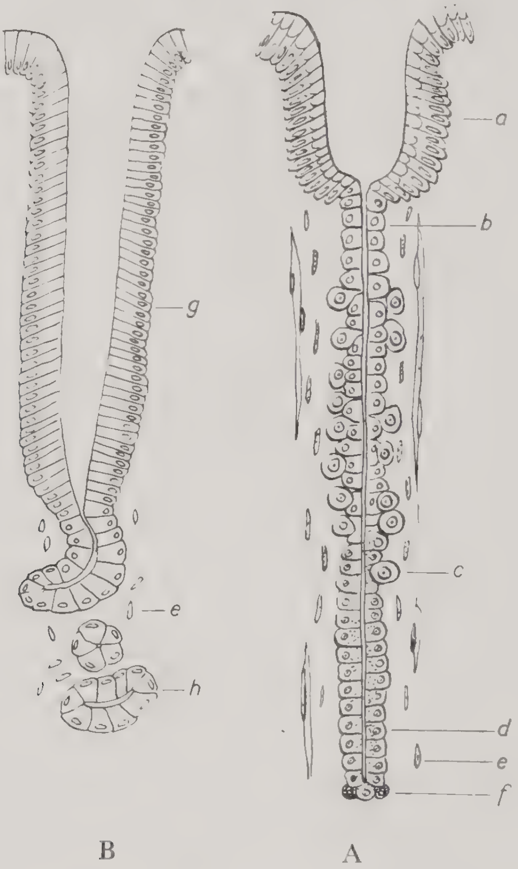


FIG. 11.1.

The various cells which produce "mucin" and related substances in the gastric mucosa. Those in italics are known to liberate "mucus" or related substances into the surroundings, i.e. the stomach contents or the intercellular spaces of the mucosa. (A) Body of the stomach. (B) Pyloric region.

(*a*) *Surface cells of fundus.* (*b*) *Neck mucous cells.* (*c*) Oxyntic cells. (*d*) Peptic cells. (*e*) *Fibroblast.* (*f*) Argentaffin cells. (*g*) *Surface cells of pylorus.* (*h*) *Mucous glands.*

gastric mucosa there are certainly two types of cell which contain "muco-substances", but there may be others which are liberating polysaccharides from their surfaces in much the same way as mechanoblasts do (Fig. 11.1). Extracts of the mucosa would be expected to contain a thorough mixture; the intracellular epithelial type would probably predominate but with some of the extracellular epithelial type present also, and perhaps with some admixture from the connective tissues. The gastric juice itself being collected over a period of time might be expected to give purely epithelial mucins but with proportionately more of the type containing uronic acid (the extra-cellular epithelial type). Another exception to the generalization of uronic acid-containing polysaccharides being "extra-cellular" in origin, is the presence of "keratosulphate" in the cornea. This polysaccharide, of apparently mesodermal origin, is one of the few so far recorded which does not contain uronic acid (Meyer, Linker, Davidson and Weissman, 1953). Here again the cellular component of the cornea is probably much higher than in many connective tissues and the conjunctiva itself may also contribute to the formation of mucins, so the origin of this exception, in a rather exceptional tissue, is worth further investigation.

On a somewhat similar theme Dorfman (1958) has pointed out that the structural polysaccharides (chitin, cellulose, etc.) all have β -linkages, whereas polysaccharides produced in globules within cells (e.g. glycogen, starch, and heparin) have α -linkages. The formation of glycogen within cells is by no means evenly distributed among cells of different types, but it should perhaps be considered as a polysaccharide in a somewhat different class from those involving hexosamines and uronic acids.

Further Utilization of the By-Products of Essential Metabolism

Needless to say, the uses to which polysaccharides and their derivatives could be put, either internally or externally, must be expected to be many and various. The task of sorting out the initial metabolic processes of which the by-products are the precursors of the polysaccharides may, therefore, not be simple. The combination of polysaccharides with sulphate ions must also be mentioned in this connexion, especially as some cells (e.g. goblet cells, cartilage cells) require the sulphur to be supplied in inorganic form, while other cells (keratinizing cells and nerve cells) only use the sulphur from substances like cysteine, cystine or methionine (Jennings and Florey, 1956; Johnston and Comer, 1957; Pelc and Glücksmann, 1955). The sulphur may be combined with polysaccharides either for storage inside the cell, as for example in the heparin-containing granules of mast cells, or for liberation as ground substance, e.g. chondroitin sulphate. It may be relevant to this line of thought to recall, once again, that the absence of SO_4 from the medium of sea-urchin eggs has an animalizing action on the embryos and its presence is necessary for the formation of the endomesoderm (see p. 163) which presumably means that the more posterior vegetal cells require inorganic sulphate. Moreover, the presence of SO_4 in the medium surrounding *Naegleria* keeps the cells in the amoeboid form. The action of this ion, therefore, which in both cases seems to favour the more amoeboid form of cell activity, on the carbohydrate organization of the cells is probably not without its significance. Its action in detoxication, however, should not be overlooked, and it is interesting to note that the "animalizing action" of oestradiol on sea-urchin embryos is much greater in sulphate-free sea water than in normal sea water, perhaps suggesting that the oestradiol suppresses the endomesoderm unless it is neutralized or "detoxicated" by the sulphate (Runnström and Kriszat, 1954).

There thus seem to be sufficiently plausible grounds for believing that the production of mucopolysaccharides and mucoproteins by a cell may be not so much an end in itself as that these substances can be formed from substances which are probably obligatory by-products of the cellular metabolism and particularly that part of it which is concerned with ionic stability. Since, however, by virtue of their very specific physical and chemical properties, they often have special application as protective agents, intercellular cements, in the formation of capsules or "ground substances" or as hormonal agents, "inducing" agents, leucotactic agents and so on, they have often been turned to advantage and used in a great variety of ways. It is thus, at present, premature to attempt to sort out their primary metabolism and to speculate on which of the constituents are basic to the metabolism of the various cell types. Much of the work on mucopolysaccharides so far has little value in relation to the actual type of cell involved in the production of these substances, since most of the tissues and fluids investigated contain several types of cell or the products of several types. For example, only one type of cell in the submaxillary gland visibly contains "mucin" granules, but this does not mean that mucopolysaccharides may not be excreted by other cells in a manner not unlike the formation of ground-substance by fibroblasts. The mammalian kidney contains no "mucus-producing cells" (though the kidney of lizards and fish may) yet mucopolysaccharides and glycoproteins (including neuraminic acid (Böhm and Baumeister, 1956)) are found in urine (Kobayasi, 1938) and histochemically in the brush-border of the proximal tubules (Moog and Wengler, 1952). Furthermore, gastric mucin may contain materials formed both in the surface cells and the neck mucous cells of the fundus if not also in the mouth, salivary glands, oesophagus, cardiac and pyloric regions of the stomach and perhaps also from the less obviously "mucous" cells of the gastric tubules themselves. Until more micro-methods are available, it is therefore too early to try to link particular polysaccharides with particular cells and, until that can be done, little real understanding of their biological action will be possible.

In connexion with the action of mucopolysaccharides as "inducing agents" it has now been shown on many occasions that epithelial differentiation is often dependent on influences spreading from adjacent "mesenchyme" cells. Sometimes these effects of the "mesenchyme" are more or less general, e.g. the induction of the palisade-like arrangement of ectoderm cells, so often and probably erroneously regarded as the first step in the induction of neural tissue, or the formation of cysts or tubules in kidney epithelium. At other times the differentiation appears to depend on more specific influences, such as are found, for example, in tissue cultures of the salivary glands where the specific pattern of the gland only occurs, or occurs most readily, under the influence of its own capsular mesenchyme (Grobstein, 1953) (Fig. 11.2). This sort of behaviour would be quite consistent with the action of the mesenchyme being dependent on exudations from mesenchyme cells of mucoid substances of greater or less specificity; for this reason it is particularly pertinent to find that the inducing powers of the capsular mesenchyme towards the submandibular gland epithelium, and of the dorsal spinal cord towards the metanephric tissue (Fig. 11.3), can occur even though the two tissues in each case are separated by a "millipore" filter which does not allow any protoplasmic processes to penetrate (Grobstein and Dalton, 1957) but which after the experiment, in some cases at least, gives a localized reaction for polysaccharides in the region where the tissues have been (Grobstein, 1955a, b, 1956). It is therefore interesting to speculate whether the induction might be due to the diffusion of these substances, which may themselves be specializations produced by the utilization of inevitable by-products of metabolism. It is, of course, possible that the movement across the filter-membrane may be accomplished by readily diffusable substances such as hexosamines, uronic acids, or other relatively small and unpolymerized molecules, and that the particular and specific polymerization may occur extracellularly; it may even occur under the influence of the cells of the "induced" tissue itself working on the particular concentrations of the diffusible substances which are being liberated by the "inducing" tissue. In other situations (e.g. blood groups) mucoproteins and mucopolysaccharides can certainly give rise to substances of the highest degree of specificity.

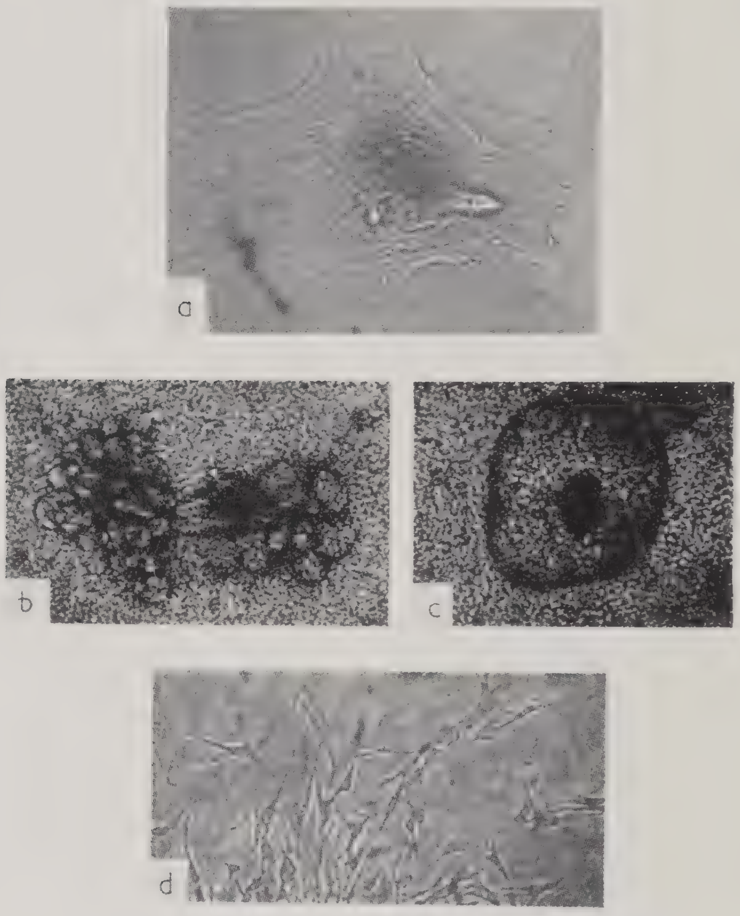


FIG. 11.2.

- (*a*) Isolated submandibular epithelial rudiment spreading as a thin sheet in tissue culture.
- (*b*) Submandibular epithelial rudiment differentiating into glandular tissue in contact with living capsular mesenchyme.
- (*c*) Submandibular epithelial rudiment failing to differentiate in contact with dead capsular mesenchyme.
- (*d*) Mesenchyme cells growing from capsular mesenchyme (Grobstein, 1953).

It should, however, be clearly stated at this point that the case for mucoid substance being the actual inducing or activating agents has not been proved, and it is of course theoretically possible that the particular tissues which act as activating or inducing agents may do so not so much by adding a positive stimulating agent to the environment of the cells which they activate, as by depriving them of some necessary metabolite, by changing their local environment in some other way or even by causing actual inhibition of some aspect of their normal activity. To consider an hypothetical case, based on the analogy with *Naegleria gruberi*, if certain cells, e.g. chorda mesoderm cells, were shown to have great powers for removing Na^+ (or other cation) from their immediate surroundings and taking it into themselves, then their activity might be such as to deprive the overlying ectoderm of its proper share of the available Na^+ and throw it over to an active Na^+-collecting metabolism. In the case of *Naegleria*, this would mean an alteration to the flagellate form, and, by analogy, the palisade arrangement of the neural plate, evoked by the chorda mesoderm, may conceivably be the equivalent response of the particular ectoderm cells to the removal of some necessary commodity of this sort.

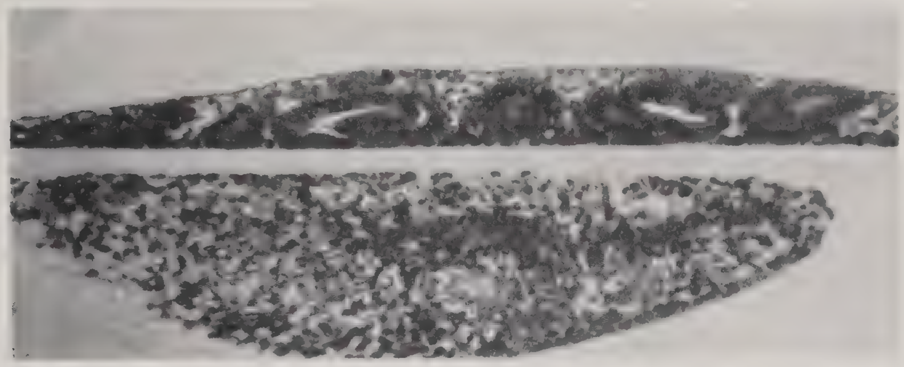


FIG. 11.3.

Epithelial tubes developing in metanephrogenic rudiment (upper tissue) as the result of the influence of spinal cord tissue (lower tissue) spreading across the separating "millipore" filter, whose pores measure about 20 A in diameter. (Grobstein, 1956).

Whether or not mucopolysaccharides, mucoproteins or their precursors are concerned with induction, it is easily seen how important a class of substances these "muco" substances are in many physiological processes. Nevertheless, the key problem of why they are so universally produced is still unsolved. Neither is it yet known how amino-sugars, uronic acids, glutamine, and glutamic acid, all of which are substances with numerous possible biochemical roles, make their basic and fundamental contributions to cell metabolism, nor if they are indeed part and parcel of the cellular processes required for maintaining ionic equilibrium in its broadest sense. Processes of acetylation and phosphorylation of the amino-sugars may also have to be considered, if these substances are important in relation to ion transport.

Finally, the association of cholinesterase with mucoproteins (Bader, Schutz and Stacey, 1944) and both acetyl-choline and cholinesterase with cell surfaces (Chang and Wong, 1933) cilia (Bülbring, Burn and Shelley, 1953; Kordick, Bülbring and Burn, 1952; Wen, Chang and Wong, 1936), and with ion transport (Koch, 1954) may be of importance in this connexion.

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CHAPTER 12

CELL FAMILIES AND THEIR ORIGIN

3. Further steps in differentiation

To return once again to the formation of mechanocytes, the picture which now emerges seems to be that certain of those epitheliocytes which originally constituted the anterior part of the embryo, or which corresponded to the primary flagellate cells in the sponge and which, because of their efforts to maintain ionic equilibrium may be assumed to be "salt-collectors" and "mucosubstance-ejectors", leave the surface and sink into the inner mass. If they there continue their same activity in relation to salt, the by-products of their ionic regulation, e.g. mucopolysaccharides and glutamic acid or other collagen precursor, must tend to accumulate within the blastocoele cavity and begin to constitute a real inter-cellular matrix from which organized connective tissues could subsequently arise. This change of position from the surface to the interior is of cardinal importance and may be the result of some change in the nature of the excreted mucopolysaccharide-precursor, for the first step in this process involves the loss of contact with neighbouring cells, and such contact and adhesiveness is itself very likely, by analogy with other tissue specificities, to have been dependent on a "mucopolysaccharide" by-product. This loss of adhesiveness and sinking-in of the cells is certainly the first main step in the differentiation of the mesohyl cells.

Furthermore if this is the method, in outline, which leads to the formation of fibroblasts, and so of collagen fibres, it means that such fibre-production may not necessarily be the complete monopoly of the mechanocytes, as such, but could result from cells, still essentially epithelial in character, but already differentiated to some extent in the direction of the mechanocyte, i.e. from pre-mechanocytes or mechanoblasts. At any rate there is evidence that the epidermal cells of the earthworm do produce a substance like collagen, though this substance is without the characteristic cross-banding of the true collagen fibre (Reed and Rudall, 1948). This would also afford an explanation for those instances, generally pathological or in tissue cultures, where a collagen-like material apparently originates from epithelial cells.

Very similar considerations apply to the sinking-in of the posterior cells to form the amoebocytes, but there is no evidence to suggest that these cells contribute in any comparable way to the intercellular mucoproteins. Indeed, the nature of their primary adhesiveness as epitheliocytes is an unsolved problem which is immediately brought into prominence by this argument.

Cell Families in Neural Tissue

If more epitheliocytes of either group (i.e. pre-mechanocytes or pre-amoebocytes) left the original surface of the blastula at a later stage, they would find within the inner mass an environment with properties somewhat different from those encountered by the earlier immigrating cells. For this reason, cells which subsequently (i.e. after the first invasion) sink in from the surface are likely to develop along different lines from those of the first generation of mesohyl cells. Their future differentiation, like that of their predecessors must depend both on the immediate effects of the new environment and on the nature of the inpushing cell, which may itself depend on the position which the cell originally occupied in the surface of the embryo. For example, the first cells which enter the mesohyl of amphibia do so from those cells which invaginate at the dorsal lip of the blastopore. They have the immediate effect of modifying the behaviour of the epitheliocytes which overlie them and causing them to become potential neural tissue. The cells of the medullary plate in their turn form a neural tube from which eventually nerve cells and neuroglia cells differentiate. The cells of the neural plate thus constitute a new race of cell, with new potentialities and whose properties emerge as the result of modification of the original epithelium by underlying cells. It is likely that this race of potential neural cells is also divisible into two main branches because the original cells of the neural plate probably contain amongst them epitheliocytes of both types (i.e. pre-mechanocytes and pre-amoebocytes). Once again this is only a broad distinction based on the two extremes of behaviour, and there may well be many of the potential nerve cells whose properties are more or less intermediate. In other words, just as there are two main classes of cells which constitute the original mesohyl, so there are likely to be two main classes of neuroblasts, one comparable with the mechanocytes and the other with the amoebocytes.

In this context it is perhaps interesting to note that the cells of the dorsal part of the neural tube behave differently in several ways from the cells of the ventral part as, for example, in relation to their influence on the differentiation of other tissues. It is only the ventral part of the spinal cord which, in tissue culture experiments, induces cartilage development from somite tissue placed in its vicinity (Grobstein and Holtzer, 1955), while it is only the dorsal part which induces differentiation of tubules in metanephrogenic tissue (Grobstein, 1955). It would be exciting to know more about the nature of the differences between the dorsal and ventral halves of the neural tube that cause these effects. The dorsal part, of course, gives rise largely to sensory cells and internuncial cells, while the ventral part produces the motor cells (Holtzer, 1950); the distribution and occurrence of mitoses in the two halves is also quite different (Hamburger, 1948). Finally, in the frog embryo, the neural tissue is formed from the neural plate and the bordering neural folds, and the cells of the former are usually columnar, while those of the latter are, for a time, also ciliated (Assheton, 1896). The neural-fold cells presumably contribute more heavily to the dorsal part of the neural tube while the neural plate contributes more to the ventral region. Some indications of two types of neuroblasts can also be obtained by studying the behaviour of isolated neuroblasts from embryos as Holtfreter (1947) has done in amphibia. Certainly the study of their movement (Fig. 12.1) and pigmentation is very illuminating.

The influence of the two parts of the neural tube on cartilage formation and on metanephric tissue differentiation respectively must, of course, also be considered in relation to the development of "fields" within the embryo. The neural plate comes into the axial field, while the folds are more lateral and overlie the potential nephric tissue. The behaviour of these tissues *in vitro* may thus be directly related to their behaviour *in vivo*. Nevertheless the "fields" must also be considered as expressions of the effects of the gradient systems in the embryo on the distribution of the cells of the primary types.

All that has been said about nerve cells must also apply to the supporting cells of the nervous system, the neuroglia and the cells of Schwann, because these cells are all derived either from the neural tube itself or from those cells which just fail to be incorporated in the tube and constitute the neural crest.

In an earlier chapter (see p. 109) evidence was presented which was certainly consistent with the idea of two main types of behaviour both on the part of nerve cells and also on the part of neuroglia. On functional grounds also, it has been suggested that the nervous system is perhaps based on an arrangement of more or less alternating neurones of different types, namely, cholinergic and non-cholinergic neurones (Feldberg and Vogt, 1948) and in the autonomic nervous system the post-ganglionic neurones are, of course, rather sharply subdivided into adrenalin-producers and acetylcholine-producers; and they have their associated satellite cells.

On these grounds, therefore, it would seem that the nervous system could be regarded as resulting from a second phase of differentiation on the part of the epitheliocytes which are already divisible into two classes. Just as, early in development, epitheliocytes leave the surface to become mechanocytes or amoebocytes according to their primary differentiation, so at a later stage both neuroblasts and neuroglia result from a secondary tissue-differentiation, and the constitutent cells in each case inevitably fall mainly into two categories because of their original constitution.

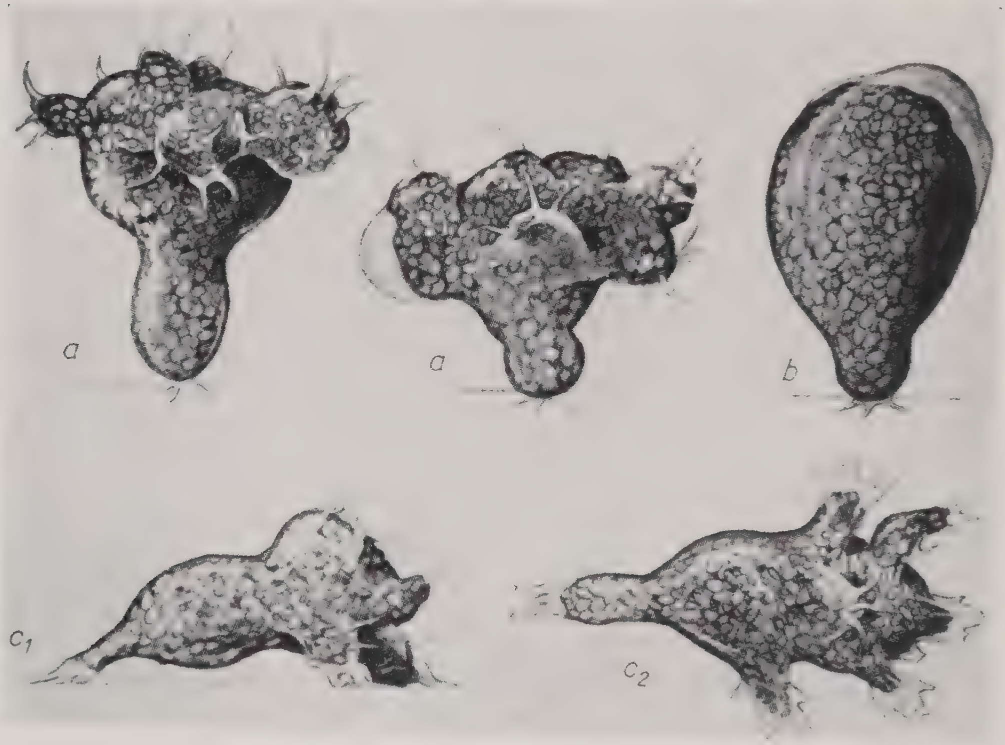


FIG. 12.1

Isolated amphibian neuroblasts.

(*a*) Changes of amoeboid form in an isolated neuroblast standing upright on its posterior pole. (*b*) Neuroblast with a rotating hyaline bulge instead of tapering pseudopodia

c_1 Migrating neuroblast seen from the side. c_2 The same cell seen from above. (Holtfreter, 1947).

An important feature of the primary differentiation in the embryos of primitive metazoa into mechanoblastic and amoeboblastic epithelial cells is the fact that these two types of cells, by virtue of a sort of symbiosis between them, initially constitute a viable organism with powers of dispersal. The one type of cell may have assisted the other in regulating the ionic composition of the organism and the fluid content of its central cavity. In addition, the anterior cells mainly provided the sensory and the flagellate motor mechanism for the more phagocytic and amoeboid posterior cells, whose chief activities were probably related to the commissariat and whose sensory and motor functions, though not negligible, were less important at this stage. Once established, this symbiosis with its advantages for ionic balance, dispersal and nutrition seems to have persisted and indeed continues to pervade the whole organism. Among the cells of the nervous system it hardly needs emphasis that there is an extraordinary degree of interdependence between the cells of different types, e.g. between Schwann cells and nerve fibres, between satellite cells and ganglion cells, and between neuroglia cells and nerve cells proper. In the sense organs, too, receptor cells are frequently intermingled with "supporting" cells. This can be seen in the olfactory organ, taste buds, auditory and vestibular organs, and even in the retina, if the pigment epithelium is thought of as "supporting" the rod and cone cells. In tissue culture the symbiosis between oligodendroglia and certain neurones persists to a remarkable degree (Hogue, 1947, 1950) and, *in vivo*, oligodendroglia and astrocytes appear to be responsible for the myelination of the nerve fibres within the central nervous system (Luse, 1956b). An obvious query is again raised here as to why there are both oligodendroglia and astrocytes. Could oligodendroglia be mechanoblastic and astrocytes be amoeboblastic in origin and do they form symbiotic pairs with, and cause the myelination of, specific types of neurones? It has already been noted (p. 110) that in the peripheral nervous system there are two types of behaviour among Schwann cells possibly pointing to a similar relationship.

Consideration of "avoidance reactions" in primitive organisms suggests that most, if not all, cells probably have within them some potentiality for transmitting the effects of a stimulus applied at one point on their surface to all other points of the surface. This property is presumably the primary basis for all forms of neural transmission; this being so, there is nothing inherently improbable in supposing that both mechanoblasts and amoeboblasts could, by suitable modification of their structure, take on the business of neural transmission, but in doing so they would probably, because of the different properties of their surfaces, adopt somewhat different means and, in consequence, show somewhat different properties. Moreover, and again because of differences in their surface membranes, the modes and rates of conduction might well be widely divergent. On this hypothesis, some nerve cells (bipolar?) may have arisen from mechanoblasts and formed a symbiotic relationship with neuroglia developed from amoeboblasts (probably the astrocytes and Schwann cells of type B), and other nerve cells (multipolar?) may have resulted from the specialization of amoebocytes as conducting agents and these cells are nurtured and stabilized by neuroglial cells derived from mechanoblasts, i.e. the oligodendrocytes and Schwann cells of type A.

This idea of symbiosis of opposite cell types does not, of course, exhaust the possibilities. Recent cinematograph studies by Weiss (1958) emphasize the manner in which cells of one type associate together, when cells isolated from two or more different epithelia are placed in the same medium; and so it is not impossible that a neural mechanoblast may associate with a neuroglial cell derived from the same stock but which has specialized in a different way from the neural cell which it supports. Both systems might have advantages in protecting and maintaining the surface of the neuron in a relatively more stable environment than that of the tissue fluid, thereby allowing for greater delicacy and sensitivity in the conducting mechanism.

The neural tube is a system which is in some ways comparable with the blastocoele cavity, especially in that the composition and volume of its contents must, of necessity, be regulated, and, until the more or less elaborate systems of the choroid plexus were evolved, it is probable that the onus of ionic regulation fell on the cells lining the whole tube, as it may still do to some extent. This would seem to be an added reason for searching for two forms of cellular activity among the potential neural tissues, the one to balance the other. Certainly the cells of the choroid plexus fall into two cytological categories (Luse, 1956a; Millen and Rogers, 1956) (Fig. 12.2).

Cell Families in Muscular Tissue

The origin of muscle cells offers a somewhat similar problem. All cells have some power of movement, and, before postulating the development of entirely new systems, the possibility that this primary mobility of cytoplasm may be the starting point for the highly elaborate muscular systems should first be investigated. In this connexion the observations of Kriszat (1950) and of Loewy (1952) on the effects of adenosine triphosphate (ATP) on the viscosity of amoebae and on that of extracts of the cytoplasm of myxomycetes are interesting. So also are the observations of Hoffmann-Berling (1954) and Hoffmann-Berling and Weber (1953) on the contraction produced by the application of ATP *in vitro* to "fibroblasts", isolated from various sources and extracted with glycerine. All these observations, in fact, point strongly in the direction of the belief that the essential, ATP-sensitive actomyosin system, characteristic of muscle, is present, in rudimentary form at least, in these primitive organisms and in the "simplified" mechanocytes of tissue cultures. This being so, there need be no monopoly on the part of any one type or family of cells in the matter of being potential source-material for muscle formation, unless it can be shown that this primitive system, which is probably responsible for cell movement, has been secondarily lost. It is thus necessary to envisage that muscle cells may develop from either amoeboblasts or from mechanoblasts. The contraction process may be similar in both cases but other features could well be different and this would naturally include the method of excitation, the histological structure and the behaviour of the cells in tissue culture. Contractile cells, in man, for example, are known to arise from a variety of sources including epithelial cells (e.g. the sphincter and dilator pupillae) and to have a variety of properties. For this reason it is probably unwise to regard muscles as a homogeneous group of tissues, but rather they should

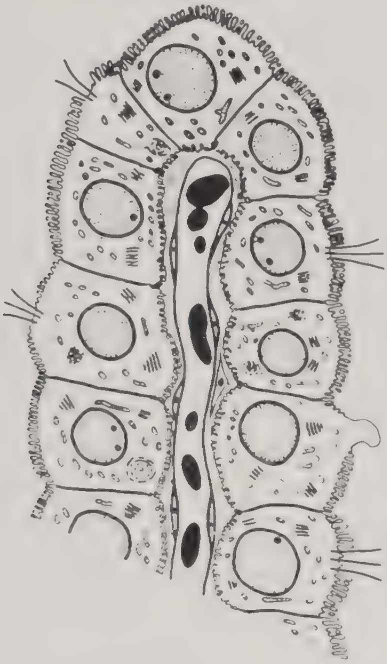


FIG. 12.2

Diagram of the two types of cell in the choroid plexus. (Millen and Rogers, 1956).

be considered as a mixed bag of tissues which have all specialized the actomyosin—ATP system to a greater or less extent within the framework of what may otherwise by essentially mechanoblast or amoeboblast.

Certainly in many muscles, even in the highest vertebrates, fibres of two types are clearly distinguishable. In the frog, one type, the "fast" type, is generally innervated by single end-plates connected with rapidly conducting nerve-fibres (8-40 m/sec). It gives rapid twitches, has a resting potential of 90-95 mV and shows an "all-or-none" propagated impulse when stimulated. The other type, the "slow" type, has multiple innervation from slowly-conducting fibres (2-8 m/sec), has a lower resting potential (60 mV) and the stimulus is only propagated for short distances over the fibre; summation effects, enabling the whole fibre to participate in contraction are produced by multiple stimulation of different units. Tension, once developed, may be sustained over long periods (Kuffler and Vaughan Williams, 1953a and b). Though these physiological properties have, so far, only been found in the frog and not in mammals, the muscles of the rat and cat have been shown to contain two types of fibres both in flexors and extensors (Gordon and Phillips, 1949; Nachmias and Padykula, 1958). One type, probably that characterized by slow contraction, has granular and lipid-containing cytoplasm (Fig. 12.3), and high succinic dehydrogenase activity (Wachstein and Meisel, 1955) (Fig. 12.4). Very often myoglobin is also a conspicuous constituent (red fibres). The other type, specialized for quicker contraction, has larger fibres and a relative paucity of the constituents

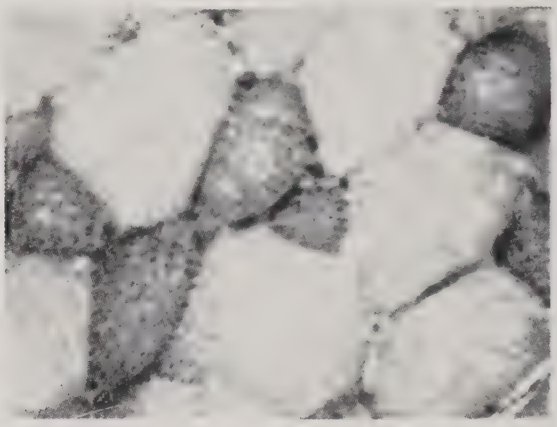
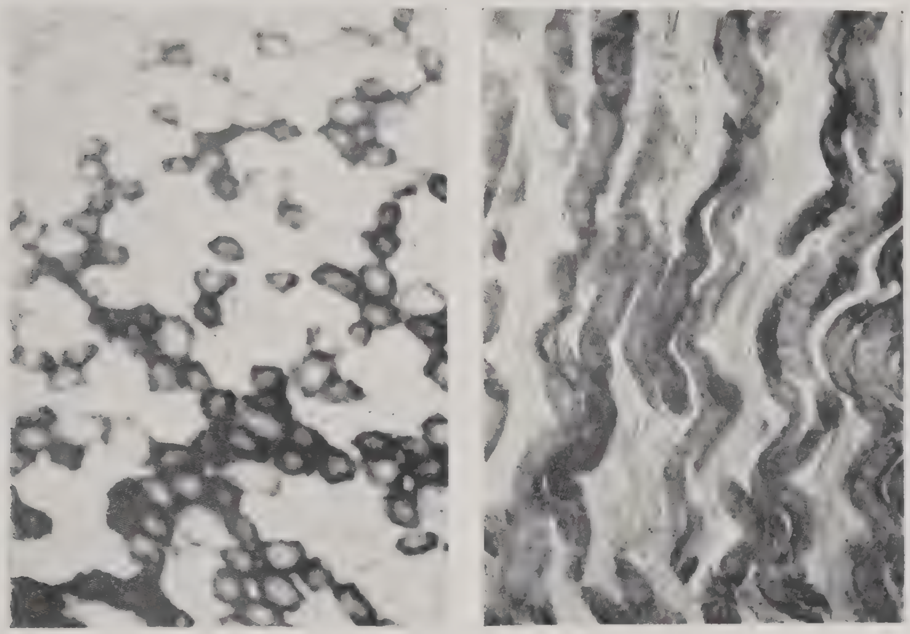


FIG. 12.3
Localisation of lipoid (Sudan-staining) material in certain muscle fibres of the rat. (Nachmias and Padykula, 1958).

mentioned. Staining with the periodic acid -Schiff reagent, after treatment with saliva to remove glycogen, also differentiates between two types of fibre (Fig. 12.5). Some fibres give a strong reaction for "esterase" while others are negative (Fig. 12.6). Apart from some ATP-ase activity which is associated with the mitochondria, both types show similar amounts of this enzyme, and this probably means that they contain similar amounts of myosin (Nachmias and Padykula, 1958).



(*a*) (*b*)

Fig. 12.4

Muscle fibres stained to show the presence of succinic dehydrogenase. Note that some fibres are heavily stained while others are almost negative.

(*a*). Transverse section. (*b*). Longitudinal section.

(Wachstein and Meisel, 1955).

In the frog, there is a further difference in structure which has been observed with the electron-microscope and which is probably related to the slow and fast fibres. Some fibres with well-differentiated myofibrils and numerous mitochondria have a well-developed system of very fine tubular canals ramifying in the region of the Z-bands and probably connecting with the surface of the fibre (Fig. 12.7). In other fibres, probably the "slow" fibres in which the myofibrils are less distinct, this system is less highly organized (Huxley, 1958). Krüger (1952) has also called attention to the distinction in a wide variety of animals, between tetanic, or twitch, fibres, which have a very clear myofibrillar structure, and slow or tonic fibres in which the myofibrils are not so individually arranged but which in transverse section yield a characteristic "field-structure" (Fig. 12.8). Krüger also quotes figures which are of particular interest from the point of view of the present argument, for they show that the tonic fibres have a much higher cation content than the tetanic fibres with fibrillar-structure (155:100). This is precisely the sort of difference which would be expected if one type of fibre were derived from mechanoblasts and the other from amoeboblasts. The whole problem would repay more detailed investigation, with particular reference to the properties of individual fibres, since most of the observations so far have either dealt with whole muscles, in which case conclusions have been drawn on the basis of whether the whole muscle was mainly red or white, tetanic or tonic, slow or fast; or else they have dealt

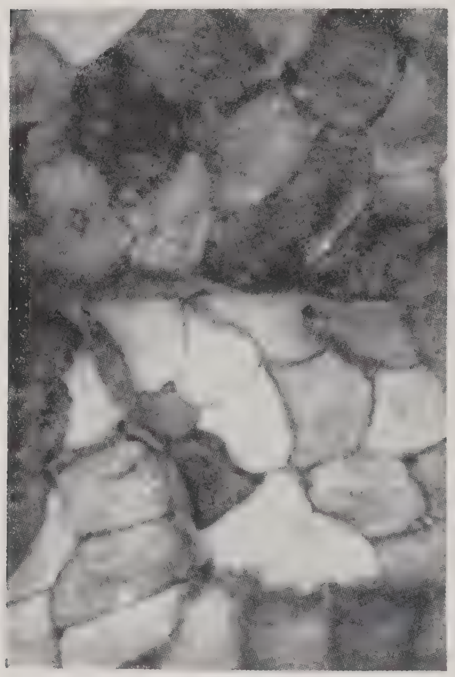


FIG. 12.5

The periodic acid-Schiff reaction on a transverse section of rat's (*Biceps femoris*) muscle, after treatment with saliva to remove glycogen.

(Nachmias and Padykula, 1958).

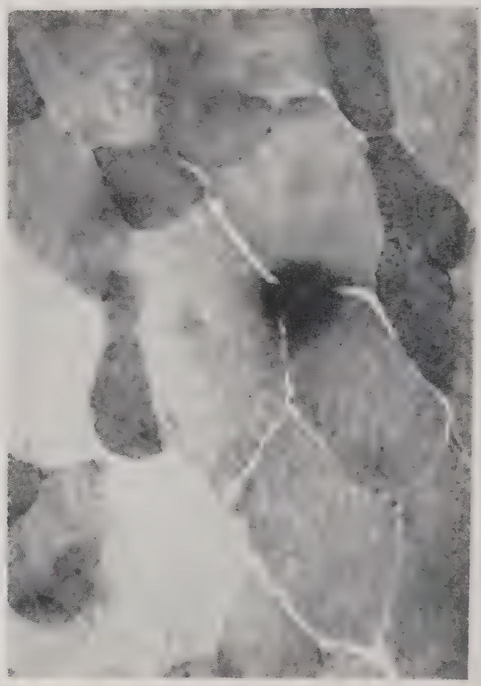


FIG. 12.6

Transverse section of rat's muscle treated with α-naphthyl acetate to show the presence of "esterase". Note irregular distribution of the 'esterase' among the fibres.

(Nachmias and Padykula, 1958).

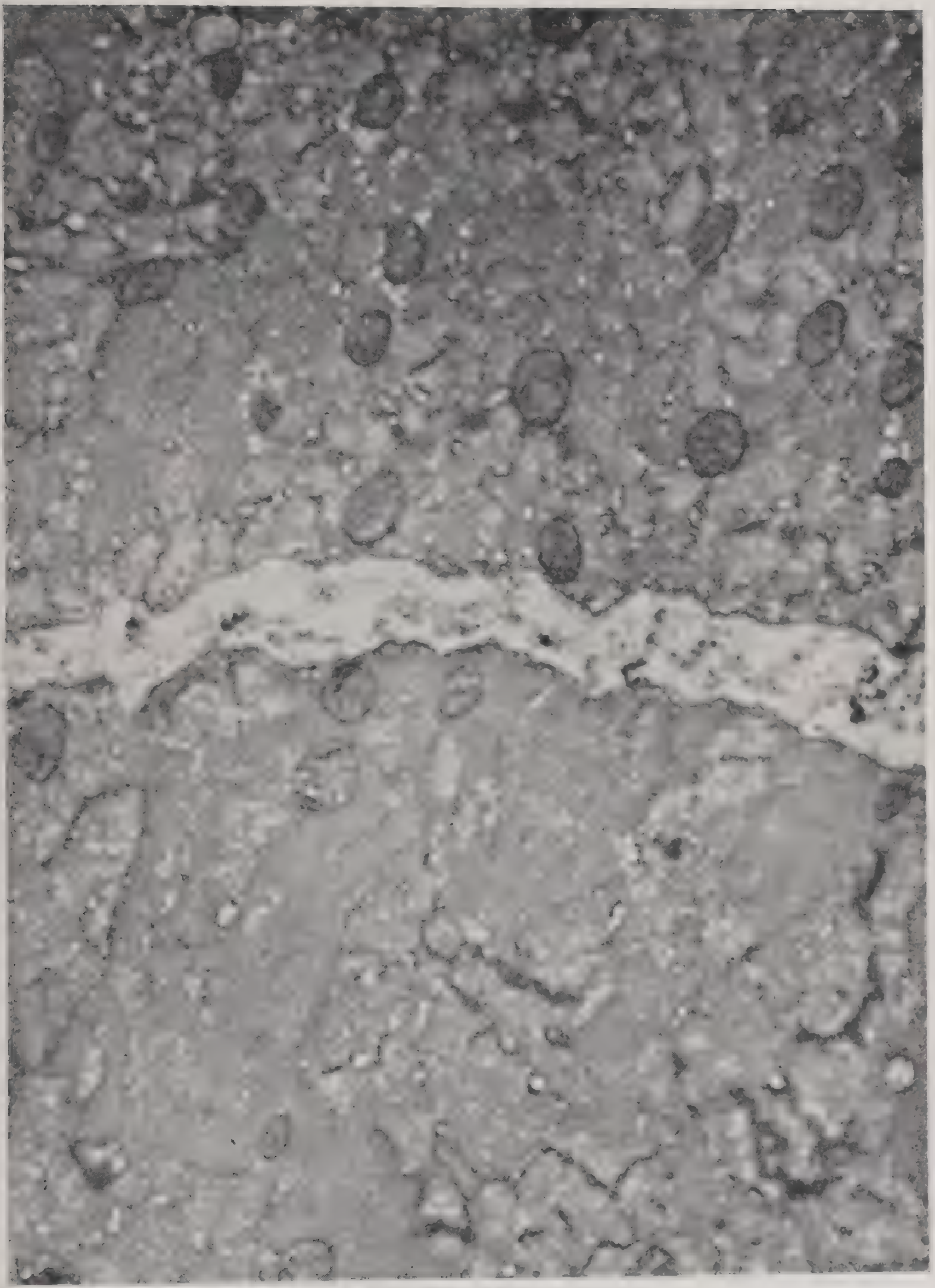


FIG. 12.7

Intracellular canals between the fibrils in frog's muscle. Note the smaller myofibrils, greater number of canals and more numerous mitochondria in the upper fibre. Compare the structure of these two fibres with those in Fig. 12.8. (Huxley, 1958).

with certain aspects of the cytochemistry of individual fibres without much reference to their particular type of physiological activity.

Greenbaum and Young (1953) have shown that different muscles respond differently in varying the amount of sarcoplasm (as distinct from the contractile fibrillar protein, actomyosin) which they contain under the action of different concentrations of growth hormone or during starvation. For example, the soleus, a red muscle, does not appreciably change its sarcoplasm under the influence of the growth hormone or during inanition. Many other muscles increase their protein content when the animal is treated with growth hormone, and lose protein during starvation. It would be interesting to know how far these changes can be correlated with the histological structure of the various muscles or with their other physiological properties.

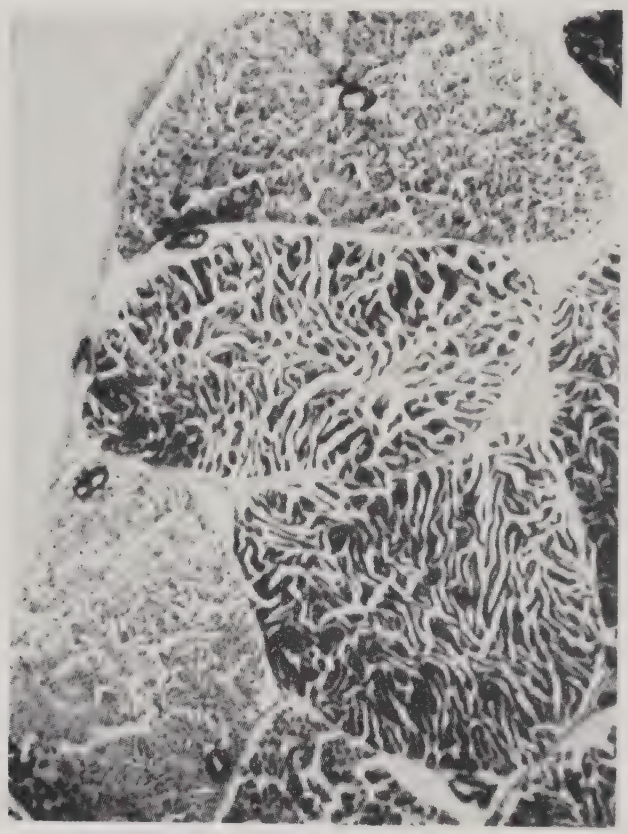


Fig. 12.8

Transverse section of muscle fibres showing "Fibrillenstruktur" and "Felderstruktur". (Krüger, 1952).

Two types of muscle fibres are also present in certain muscle spindles in the cat. There are large and long fibres with a concentration of nuclei near the annulo-spiral nerve-endings and there are also smaller and shorter fibres each with a chain of nuclei down the centre. The large fibres have motor-end-plates of the usual pattern, but the small fibres have extensive endings of quite a different type, possibly similar in kind to the endings on the slow or tonic fibres of lower animals (Boyd, 1958). Here again, therefore, there is a suggestion of two essentially different types of muscle fibre, and, what is more, they are supplied by nerves of two different types.

Red and white fibres in the cat have been found to differ in their sensitiveness to adrenalin (Bowman and Zaimis, 1955) and to transmission-blocking agents like decamethonium (Jewell and Zaimis, 1954).

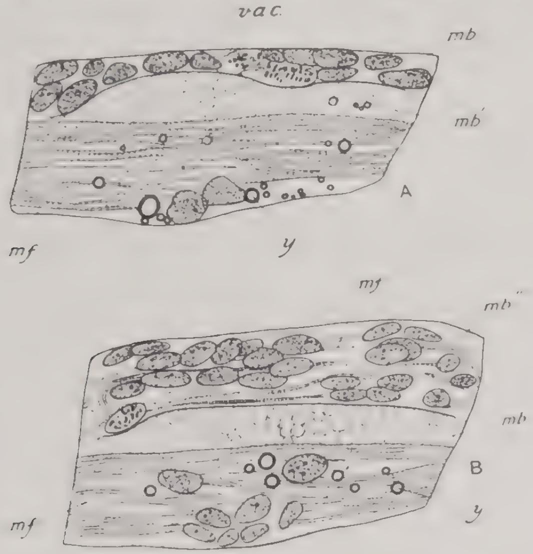


FIG. 12.9

Differentiation of the myotome as seen in horizontal sections of *Lepidosiren* larvae. B represents a later stage than A

mb′.	myoblast of inner wall
mb″.	myoblast of outer wall
mf.	contractile fibrils
vac.	vacuoles, y. yolk.

(Kerr, 1919).

Furthermore, Kerr (1919) called attention to the different ways in which the striations appeared in the muscle fibres developing in the two parts (sclerotome and dermatome) of the myotomes of *Lepidosiren*. On one side the individual spindly myoblasts each acquire their own striations: on the other side a more or less syncytial mass develops its striation in one part of a granular yolk-ridden cytoplasm (Fig. 12.9). It would be interesting to know the further fate and properties of these particular muscles, and to know more of the origin of "slow" and "fast" fibres in general.

All these various observations begin to amount to a rather suggestive body of evidence in favour of the view that there may be muscle fibres of amoeboblastic origin as well as of mechanoblastic origin, and that while the actual contractile mechanism may be similar in the two forms, other differences, akin to those between "fibroblast" and "macrophage" may well exist in different muscle fibres. Presumably such a duality among the muscle fibres would be intimately related to the duality in the nerve tissues that has already been suggested, and certainly the observations of Boyd (1958) and others indicate something of that kind. There is indeed some evidence that the type of fibres which develop in a muscle depends on the type of nerve fibre which innervates them. Such an interpretation of muscle would accord well with the frequent appearance of multinucleate cytoplasmic masses, comparable in many ways with the giant-cell formation so commonly observed among amoebocytes (Fig. 12.10), and of the differing behaviour of fibres in tissue culture whereby some split up much more easily than others and tend to produce mechanocytes. (Fig. 12.11).

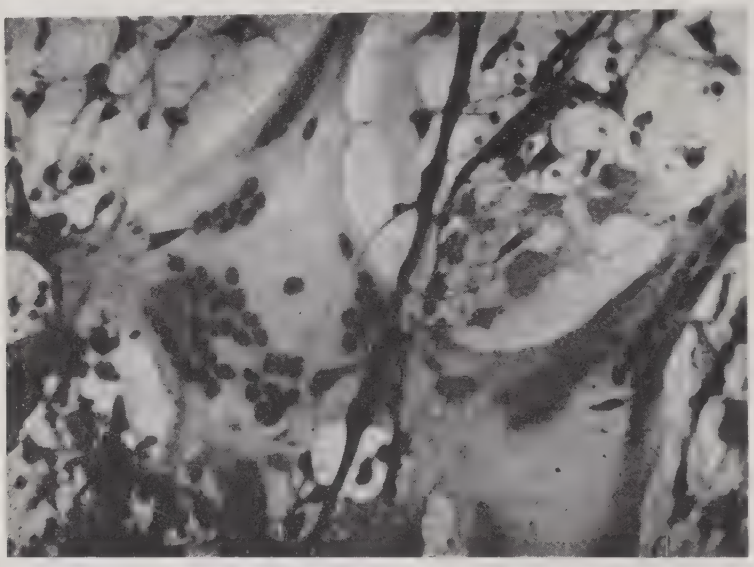


Fig. 12.10

Multinucleate giant-cell mass developing from a muscle fibre.

(Lewis and Lewis, 1917).

Cell Families in Surface Epithelia

The cells which are finally left in the surface layers of the organism, i.e. those which have not entered either the primitive mesohyl, or become neuroblasts and neuroglia, or developed into muscles at a later stage, presumably still retain something of their primary duality, and it would therefore be expected that in most epithelia there must, at least potentially, remain two sorts of cells in varying proportions, one still bearing traces of its origin from the anterior or animal pole of the "embryo" and the other reflecting its origin among the posterior vegetal cells. It will be remembered that in an earlier chapter some stress was laid on the observation that most epithelia bear more than traces of having cells of at least two categories; the cause of this duality may lie in the initial constitution of the epithelium from blastomeres of two types.

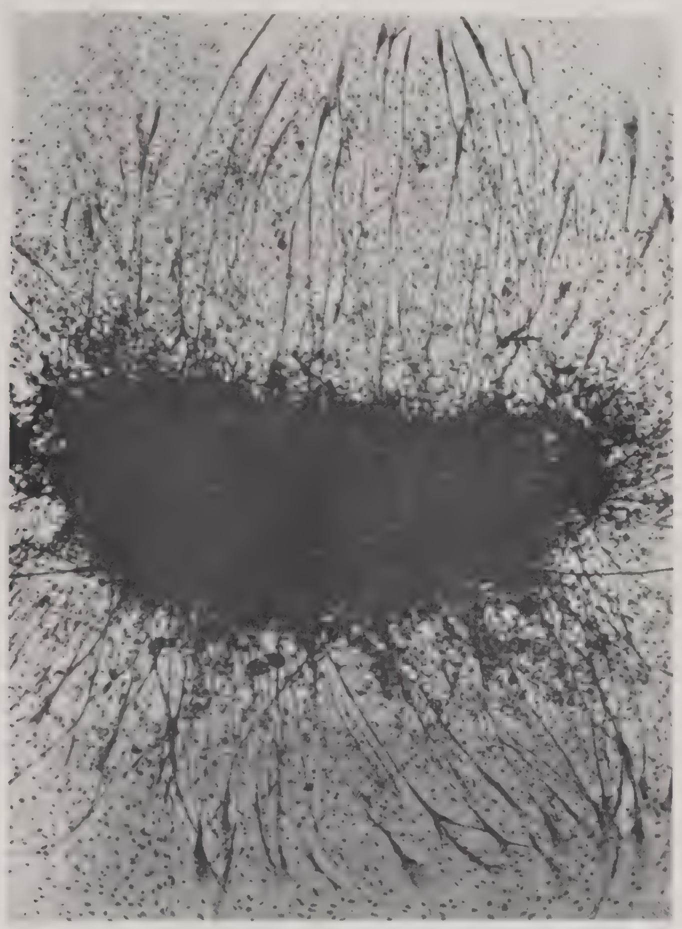


FIG. 12.11

Culture of skeletal muscle showing multinucleate muscle buds developing from some fibres, and a copious outgrowth of mechanocytes occurring at the same time. (Lewis and Lewis, 1917).

Further study of epithelial cells, particularly with the assistance of the electron-microscope, seems to bear out the essential truth of this observation and at the same time may clarify some of the difficulties which arise when too strict a dichotomy is postulated. Electron-microscopy of various simple epithelia, i.e. epithelia consisting of only a single layer of cells or with only a further layer of basal embryonic cells, seems to reveal two extreme forms of cell, represented in different epithelia, with a graded series of intermediate forms. The relevant structural features of these cells are exemplified in the diagram (Fig. 12.12) and attention is drawn to the two surfaces of "interchange", i.e. the distal, luminal or free surface of the cell and its proximal or basal surface, i.e. the surface nearest the blood capillaries. Each of these surfaces may show some degree of independence of the other. Of the epithelia which have been studied so far several contain at least two members of this series (Figs. 12.2 and 12.13) and there is certainly some correlation between the form of the cell and the salt-equilibrating mechanism required of it (Pease, 1956), if the basal surface of the cell is assumed to be always in contact with a relatively stable environment, e.g. tissue fluid. The various forms probably constitute recognizable steps in a graded series showing maximum water conservation with perhaps cation-ejection at one end of the series (*a*) and maximum water loss, with ionic conservation at the other (*e*) (see Fig. 12.12). In the respiratory epithelium, for example, which may be subjected either to the danger of desiccation on the one hand or of water intoxication on the other, there are cells at the two extremes ready to deal with the situation. There are the ciliated cells which are probably "the water-ejectors" (and salt-retainers) and mucous cells which by analogy could be regarded as "water-retainers" (and salt-ejectors). In nephridia there are flagellate water-ejectors, the activity of whose flagella increases with reduction of the ionic content of the medium (Roots, 1955) and absorptive cells probably akin to (*b*), while in the kidneys of the higher animals where the environment of the cells is on the whole more closely regulated, the two main cell types (proximal and distal tubule cells) seem to centre round classes (*b*) and (*d*) (Pease, 1955) (Figs. 12.14 and 12.15). In the choroid plexus the cells are of types (*c*) and (*e*) (Millen and Rogers, 1956) (see Fig. 12.2).

On these lines not only the epithelial cells but also the cells of the inner mass or mesohyl could be thought to constitute a continuously graded series with mechanocytic qualities more developed in cells coming from epitheliocytes (*d*) and (*e*) and amoebocytic qualities in those coming from types (*a*) and (*b*). It might be very difficult to change an (*a*) cell into an (*e*) cell but less so to change a (*b*) into a (*d*), and a (*c*) cell could perhaps be easily persuaded to display some of the properties of either (*b*) or (*d*), or even of both, though if the main difference is

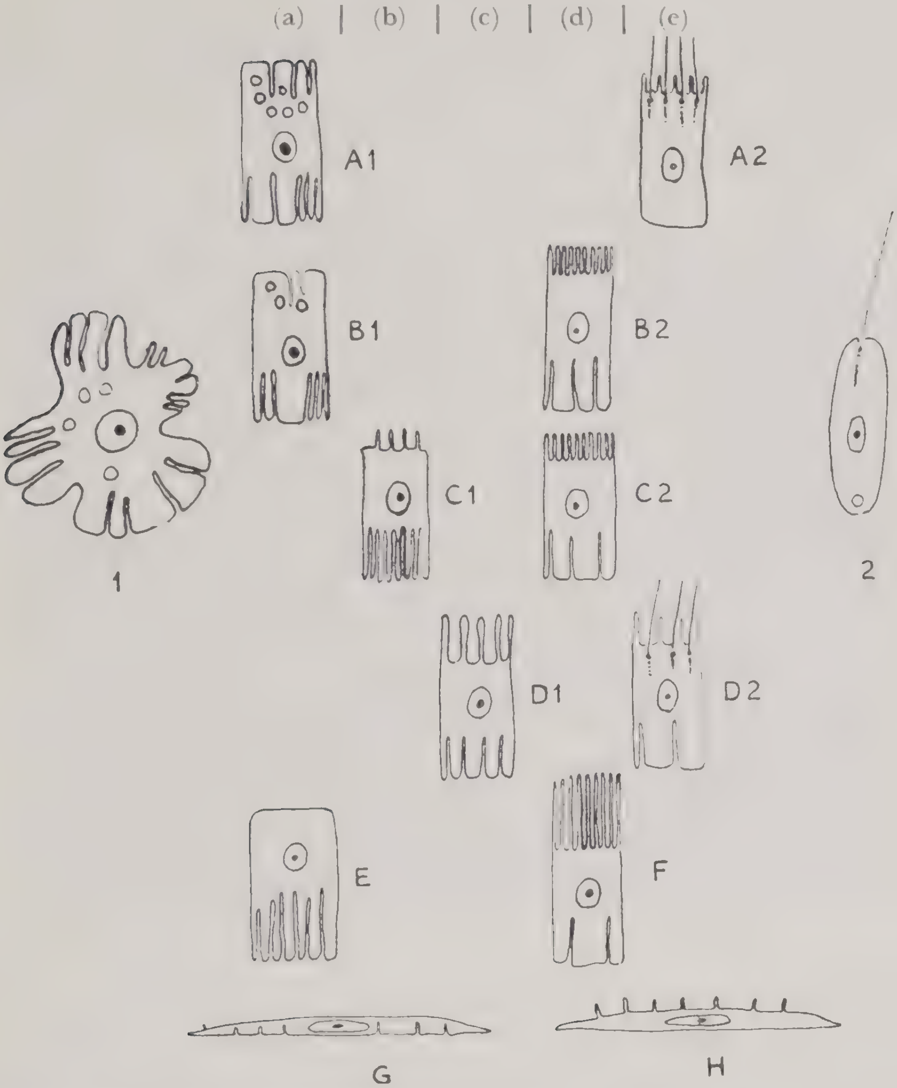


FIG. 12.12

Simplified diagrams of electron-microscope structure of cell membranes, showing the distribution of cilia, microvilli and infolded membranes. The interdigitations with neighbouring cells have been omitted. In the epithelial cells the upper surface is the free or distal surface and the lower surface is the basal or proximal surface adjacent to the tissue fluids. In E the infolded membranes lie next to the aqueous humour from which they are separated by a "basal membrane".

1. Amoeba
- (a) Trachea
  - 1 Goblet cell
  - 2 Ciliated cell
- (b) Intestine
  - 1 Goblet cell
  - 2 Brush-border cell
- (c) Kidney
  - 1 Distal tubule cell
  - 2 Proximal tubule cell

2. Flagellate
- (d) Choroid plexus
  - 1 'Polypoid' cell
  - 2 Ciliated cell
- (e) Ciliary epithelial cell from the eye
- (f) Epididymis cell with 'stereocilia'
- (g) Endothelial cell
- (h) Cell from peritoneal surface

related to directional movements through the surface, any part of the surface would presumably be orientated in either one direction or the other. How far the surface of a cell in a uniform environment can exist as a mosaic is a moot point, but, to some extent at least, it certainly can.

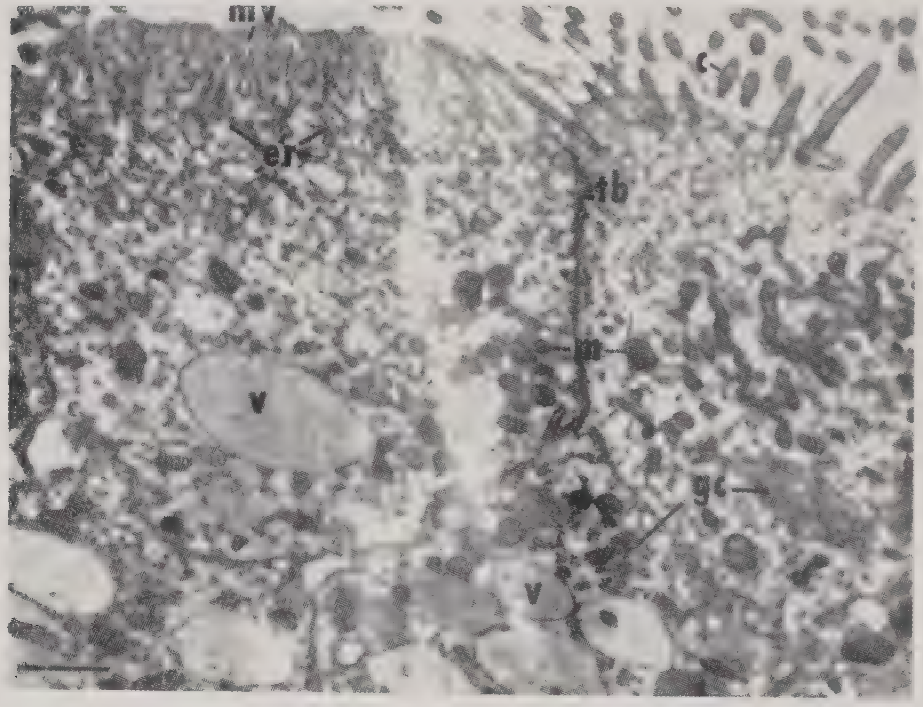


FIG. 12.13

Two types of cell in the ductulus efferens of the guinea-pig, one with microvilli (mv) and the other with cilia (c)

er.	endoplasmic reticulum
tb.	terminal bar
gc.	Golgi complex
v.	vacuole
m.	mitochondria

(Ladman and Young, 1958)

Since these cell-pairs normally exist side by side in the epithelia and tissues of higher animals, it follows that the differentiation has reached a fairly permanent state and that, in general, environmental influences do not cause metaplasia as they may in simpler organisms. When the environment does apparently lead to changes in the proportions of cells in a tissue in the higher animals, it is probably by cell death and preferential replacement rather than by conversion. For this reason the primary subdivision of the cells of an organism into two main camps, with intermediate forms, remains of basic importance in relation to the differentiation of all cells, and it is from this primary dichotomy that all subsequent patterns of differentiation emerge.

The morphogenetic movements during embryological development are probably sufficient to account for most if not all of the mingling of the two primary cell types which must occur. Such morphogenetic movements may take place either in the mass, as in the formation of the primitive streak, the neural tube and the somites of the bird embryo or in the formation of the keel of the sternum (Fell, 1939; Chen, 1952), or, alternatively, smaller groups of cells and even individual cells may wander about, as, for example, the germ cells have been shown to migrate from their original position in the endoderm and to invade the coelomic epithelium of the genital ridge, or as the sympathetic ganglion cells wander well away from their original position in the neural crest, finally to come to rest in the ganglionic chain or in the medulla of the adrenal gland. Perhaps even more spectacular is the 'directed' migration of the pigment cells from the neural crest of birds as the result of which the extremely precise pattern of feather colouration develops (Du Shane, 1944). In addition to these wide and independent migrations, a slower mingling process probably goes on continuously and within well-defined limits through-

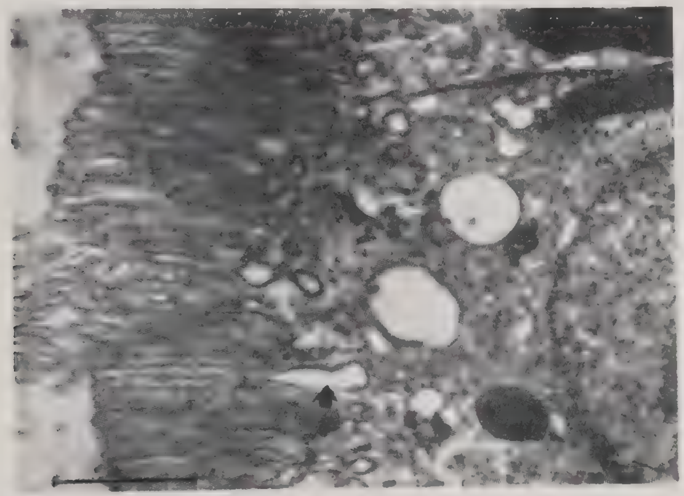


FIG. 12.14

Proximal tubule cells of the kidney; the apical region of proximal tubule cells showing "brush-border", vacuoles and intracellular canals. (Pease, 1955.)

out life. The cells in epithelia are probably always adjusting their positions; this is very obvious in speeded up films of epithelial cells growing *in vitro* where the cells constituting the typical "sheets" can be seen to be constantly creeping around each other. It also must occur in such epithelia as that covering the villi and the crypts in the intestine, even to the extent that, in the rat, the average life of a cell in the epithelium of the villus, i.e. the time between its birth in the crypt and its destruction at the tip of the villus, is only about two days (Hooper, 1956; Leblond and Stevens, 1948). Even in epithelia which are apparently more static, there is probably some continual movement. For example, in the formation of teeth an apparently simple columnar epithelium composed of one layer of cells is responsible for the deposition of the enamel. Each cell lays down an enamel prism on that surface of the cell which faces the odontoblasts, and the enamel thickens by the elongation of these prisms brought about by the addition of more matrix from the cell surface. If the formative cells secreted equally over their "free"

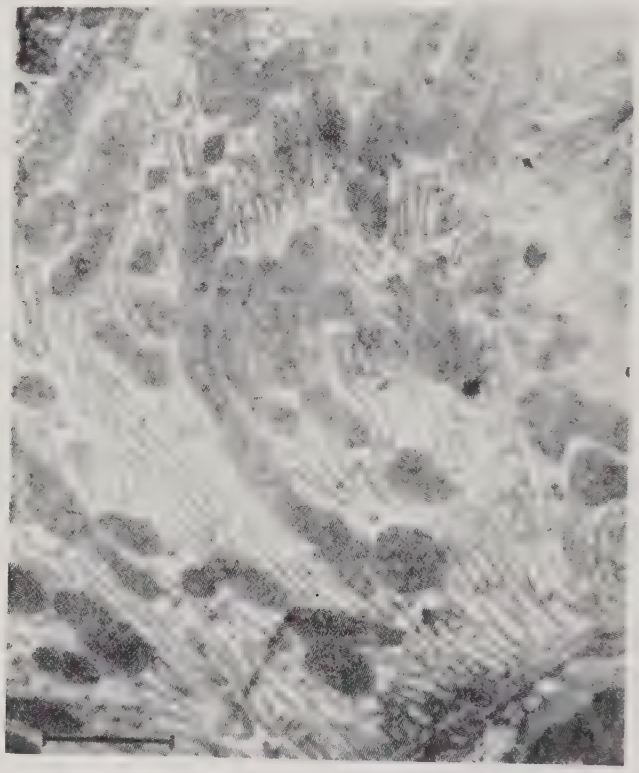


FIG. 12.15
The basal portion of distal tubule cells, cut almost parallel to the basement membrane. (Pease, 1955).

surface and if they remained in the same relative positions with respect to each other, the enamel prisms would presumably all be exactly regular hexagons in parallel arrangement. While this regular "crystalline" array is actually found in some regions of the enamel there are always to be seen other parts in which the enamel "prisms" are woven together in a criss-cross pattern, and this can best be accounted for by change in the relative positions of the formative cells (Fig. 12.16). Incidentally, the enamel organ is one of the few epithelia in which there appears at first sight to be only one type of cell, but even here there are really two types because the more superficial layers of the epithelium form that curious structure known as the "stellate" reticulum of the enamel organ where the epithelial cells have become stellate and lie in a ground substance containing mucopolysaccharide thus superficially resembling mesenchyme cells. They do not however produce collagen.

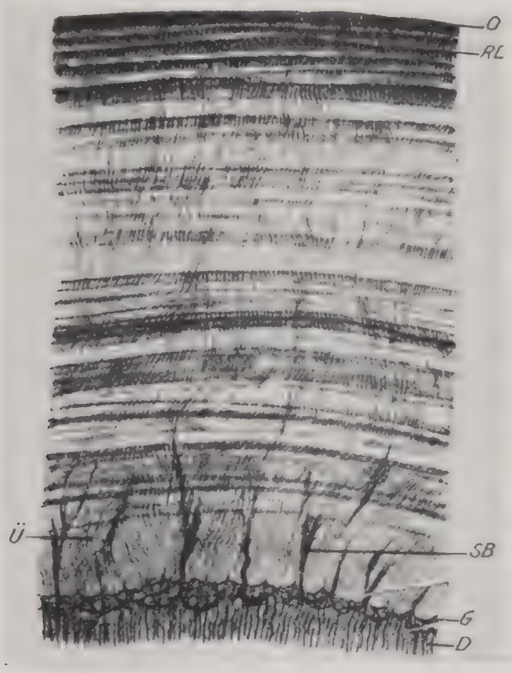


FIG. 12.16

Ground cross section of the crown of a human tooth showing the formation of the enamel prisms.

D. Dentine. G. Boundary between enamel and dentine. O. Surface of the tooth. RL. Parallel fibres of Retzius. SB. Enamel tuft. Ü. Criss-crossing of prisms. (From Maximow and Bloom, 1930, after Schaffer).

Largely on grounds of analogy but also on grounds of homology it might be expected that some epithelia, particularly those near the anterior end of the body, while possessing a mixed collection of cells belonging to the two main groups, would contain predominantly mechanoblasts, while epithelia derived from nearer the posterior end of the body or the original vegetal pole would, other things being equal, have more cells of the amoeboblast type. This general expectation as to the proportions of cells present obviously does not always hold good. During development one group of cells may at any time multiply rapidly, while another may hardly increase at all. Another group of cells may degenerate and die out. Observations on the histology of developing organs show emphatically that cell degeneration sometimes plays a very large part in embryological development (Glücksmann, 1951). In the retina of the tadpole, for example, a whole generation of cells arises and then immediately disappears (Glücksmann, 1940). Similar waves of degeneration can be seen in the development of the nuclei in the spinal cord (Hamburger and Levi-Montalcini, 1950) and in spinal ganglia (Hamburger and Levi-Montalcini, 1949) though in these cases the degeneration occurs among cells rendered superfluous by the development of definitely localized limbs.

Finally there may even be conditions in the early stages of embryonic development, while the cells are still plastic and before determination and differentiation have set in, which allow the wavering intermediate cells to change from one type to the other. Indeed, it seems likely that in many cases the cells of an epithelium may remain "on the fence" till a very late stage of development. For example, the basal cells of the respiratory epithelium appear to be a case in point. These cells normally give rise either to ciliated cells or goblet cells, which are certainly of opposite types, and the question therefore arises as to whether these basal cells are really "indifferent" or whether they number among themselves some cells that can only develop into the ciliated type and others that can only produce goblet cells. Certainly there is little evidence that, once ciliated or goblet cells have differentiated, either can revert or change to the opposite type.

It may be necessary therefore to bear in mind the possibility that animals may preserve a store of such uncommitted cells till late in development or even into adult life. If the differentiation into mechanoblast or amoeboblast depends on position in the embryo the cells in the intermediate zone would certainly be the least committed, and if such cells were placed in a stabilized environment they might remain uncommitted till some environmental change occurred. Obviously the forces at work in the differentiation of the ciliated and goblet cells of the trachea from the basal cells are worthy of a much more thorough investigation than they have so far received.

In consequence, therefore, of such factors as morphogenetic movements of cells, differential cell multiplication and differential death rates among cell groups, it cannot be expected that any tissue in such a highly organized animal as a vertebrate, upon which the forces of evolution have acted for countless generations, is likely to remain composed exclusively of either mechanoblasts or amoeboblasts, unless the one or the other has been specifically eliminated. Moreover, mechanocytes and amoebocytes differ among themselves in the extent to which their specific characters are developed; men undoubtedly differ from women, but no one will deny that there are gradations of sexuality.

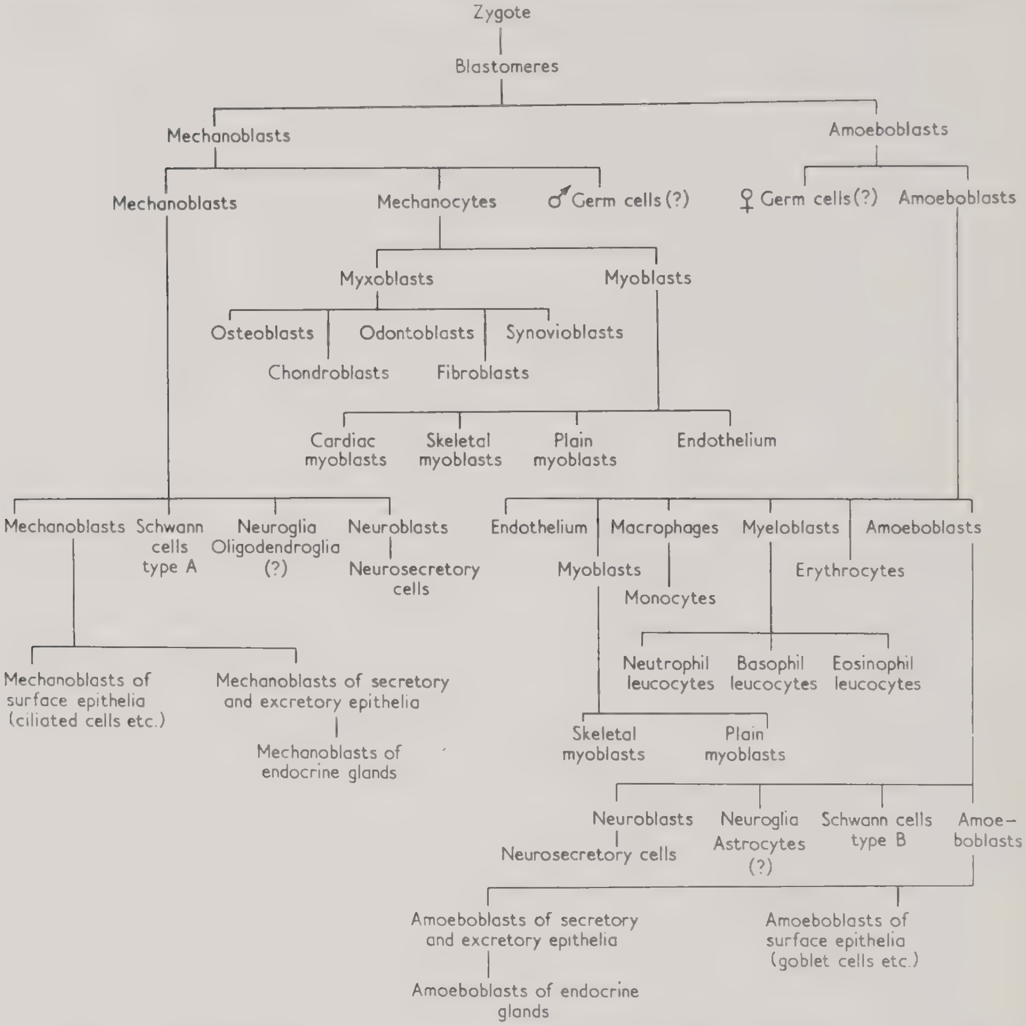
A Scheme for the Relationships between Families of Cells

Most tissues inevitably end their development as mixtures, but a knowledge of the origin of the constituent cells and of some of their latent potentialities must help in elucidating some of the curious and apparently sporadic inter-relationships between cells and tissues which occur not only within any one organ but also in different parts of the organism, e.g. in the relationships between specific cells in endocrine glands and their so-called "target" organs.

The "genealogical tree" illustrated in Table 1, shows a possible interpretation of some of the various groups of cells and tissues in terms of their origin and probable phylogenetic relationships. A study of this table emphasizes the existence of a limited number of main branches each breaking up into smaller and smaller units, in very much the same manner as do the families, genera and species of the more usual evolutionary trees. There is, however, one important difference between the evolution of the cell types in the organism and the evolution of animal species. With somewhat rare exceptions, species are in competition with each other and partnerships are exceptional. The cells of an organism, on the contrary, form a closely knit community and each is working for the good of the whole; two cells are often better than one, so that partnerships and 'symbiotic' relationships are numerous, and cells of several types often act together to achieve some physiological purpose. It has been suggested that mechanoblasts and amoeboblasts have resulted from the first of these partnerships, the one which was established in order to maintain the "ionic balance" of the primitive colonial organism.

TABLE 12.1.

A possible genealogical tree of the cell families in a vertebrate



N.B. In interpreting this 'tree' it is most important to realize that mechanoblasts do not differ absolutely from amoeboblasts, but that there is a continuous gradient from one extreme to the other. The table therefore overaccentuates the dichotomy. With appropriate changes, the table can be applied to the development of any tissue or organ.

THE EFFECTS OF LOCAL ENVIRONMENTS ON THE DIFFERENTIATION OF TISSUES

Another important feature in the evolution of cells, which has a close parallel in the evolution of species, must also be mentioned. In the evolution of species the units remain more or less independent of each other, but whole groups may be subjected to some peculiarity of their environment and may adapt themselves to that environment by similar changes. For example, it is a common feature of many groups of animals quite unrelated to each other, birds, bears, ermine, etc., that under arctic conditions they cease to produce melanin in their skins and so all become white. This apparently adaptive change is independently achieved in each case, though the basis for it may be a heritable variation which, at the cellular level, probably has a common origin, and it is evoked by the environment either directly or indirectly, presumably indirectly.

A situation comparable with this occurs in the evolution of races of cells in the animal body and actually lies at the root of the adaptive colour-changes mentioned above. As has been shown, morphogenetic movements cause considerable mingling of the cells in the course of embryonic development so that it is probable that the cells in any given region of the later embryo are already a thorough mixture. Certain regions, however, acquire special potentialities and peculiarities. These seem to arise, at least in part, by virtue of the position which the tissue occupies in the embryo. For example, the "neural crest" comprises those cells which just miss incorporation into the neural tube, and this tissue is the source of almost all those cells in the body normally producing melanin (Du Shane, 1944). The only exceptions to this are the cells that form the pigment epithelium of the retina, and a few genuine nerve cells, i.e. nerve cells developed from neuroblasts in the neural tube itself. Many of these neural crest cells, if they do not produce melanin, adopt an alternative pathway for the metabolism of dihydroxyphenyl alanine and tyrosine and they produce some substance like adrenalin. All the sympathetic ganglion cells and the cells of the adrenal medulla fall into this class. There is thus produced a geographical peculiarity of metabolism in this part of the embryo; something in the environment of neural crest cells apparently brings about this special feature of tyrosine metabolism. Indeed, there is now evidence that this special factor is the α-amino group of phenyl-alanine itself and that phenyl-alanine is the "inducing agent" for pigment formation by cells of the neural crest (Wilde, 1955). The idea of a spatial peculiarity of the neural crest region in this respect is further strengthened, because when cells from other regions of the embryo are subjected to the same stimulus in isolation *in vitro* some of them may also respond in a similar manner though they would not normally do so in the body. For example, when phenyl-alanine is added to tissue cultures of even the ventral ectoderm, the cells of which would never normally produce melanin, some of these cells can actually be induced to do so. The peculiarity of the neural crest in being the seat of melanin-formation thus appears to be partly a peculiarity in the distribution of phenyl-alanine, or some closely related substance, in the embryo. It is thus a rather similar situation to that of arctic conditions evoking whiteness in animals.

Whether this susceptibility to phenyl-alanine applies to all the cells of the neural crest equally or only those of the amoeboblast group is uncertain, and the cellular constitution of the neural crest must be examined further. Most melanoblasts undoubtedly have the general behaviour of amoebocytes as can be clearly seen in the melanophores of fishes and amphibia, in the cells of the choroid coat of the vertebrate eye, and in the dendritic cells of the mammalian skin. On the other hand the genuine pigment epithelium of the iris and retina (though not, strictly speaking, derived from neural crest) displays behaviour in tissue culture which, when not truly epithelial, is more akin to that of mechanocytes (see, however, p. 377). Other cells, e.g. the basal cells of the epidermis, and certain fibroblast-like cells in tissue cultures (melanophages (Wilde, 1955)) can pick up melanin granules from true melanoblast cells, but they probably do not form it. Sympathetic ganglion cells, or at least some of them, and cells of the adrenal medulla, are adrenalin-producers and also derivatives of the neural crest, and some of these in tissue culture show features characteristic of mechanocytes. Furthermore, neural crest cells must certainly include a number of actual or potential mechanoblasts, because, in some animals and under some conditions, they may yield fibroblast-like cells and even cells which form cartilage and teeth; at any rate, such structures do not develop when the appropriate piece of neural crest is removed. Moreover, the differentiation of these so-called ectomesenchyme cells of the neural crest can be fostered in tissue cultures by the presence of phenyl-lactic acid in the medium in place of phenyl-alanine. It is, however, inhibited by substances of somewhat similar type in which the phenol group has been replaced by a five-membered ring, e.g. thienyl-alanine, though such compounds allow the pigment cells to develop.

It seems probable, therefore, that a special phenyl-alanine or tyrosine metabolism is something which is normally peculiar to the area of the neural crest and, to some extent at least, the neural crest area is determined as much by the distribution of a phenyl-alanine-like substance as by the nature of the cells themselves in that area. The latter are cells which are either a thorough mixture of amoeboblasts and mechanoblasts, or more likely, cells not as yet committed in either direction, i.e. cells of somewhat intermediate type, as from the middle of the "gradient". Other factors may, however, also be at work in the production of pigment, since Figge (1940) has shown that the oxidation-reduction potential in a cell is critical for the functioning of tyrosinase, which is one of the key enzymes in melanin formation, and that, by altering this potential, melanoblasts may be prevented from producing melanin, while other cells can be induced to do so.

There are thus in the differentiation of cells, at least three sets of influences at work. First, there are the species-specific and genetically produced characters; second, there are characters which may be dependent initially on the environment for their appearance but which influence the cell in a way which is virtually irreversible, e.g. the mechanocyte, once it is formed from epithelium, continues to breed true as mechanocyte and cannot revert to the same type of epithelium, though it may perhaps be able to form secondary membranes of a sort; thirdly, there are the frankly reversible modulations depending on environment only. In the last two cases there are presumably some underlying genetic factors upon which the environment plays, but in the absence of the environmental factor such genetic factors may not manifest themselves, at least not in the same way. Once the environment has called forth the special response on the part of the cells, the specialized cells, unlike the animals in which the environment has produced a change in colour and which need the environment to call forth the colour, may continue to reproduce cells of their own type, e.g. melanoblasts continue to re-produce themselves and sympathetic ganglion cells divide to produce cells of the same form, whereas hares only produce white hair under arctic conditions and their offspring in temperate regions are normally agouti. Like all analogies, therefore, this one also breaks down when pushed too far.

Nevertheless the concept of families of cells, each with its own biochemical and metabolic peculiarities, is a useful one from the histological point of view and it will be seen, in the chapters which follow, that it offers certain causal explanations for some otherwise apparently inexplicable similarities and relationships between cells. These cell families, however, differ very strongly in one way from natural families of animals, etc. They depend for their emergence much more on environmental factors than on genetic factors, in the true sense of those factors that produce specific racial characters, though perhaps not in the sense of plasmagenes.

The Effects of Cell Movement

It is most important to realize that the individuals belonging to the families of cells may be scattered throughout the body, just as the members of human families may be dispersed all over the globe, though sometimes preponderating in certain areas, and this dispersal into small units makes any biochemical analysis extremely difficult. Any one organ may contain cells representing numerous families or races. An organ may be more like a cosmospolis than a family.

Up to this point most stress has been placed on the biochemical and structural aspects of cell behaviour, with some little attention given also to the type of movement of individual cells. This last is however, often a most important feature of cell activity and one which the earlier cytological methods did little to elucidate. Nevertheless, the methods of tissue culture, experimental embryology and the application of cinematography to cell and tissue physiology have shown how characteristic and important these movements may be. They have not only shown how individual cells move but have unravelled the extent and character of some of the mass migrations of cells that produce the formative movements in embryological development; they have shown, for instance, how the cells required for the development of some special feature in the animal may sometimes arise by mitosis in quite a different part of the animal, from which point the formative cells migrate in an organized fashion towards their destination. A classical example of this type of movement to which brief reference has already been made, is seen in the formation of the sternum in the chick and indeed in other animals also. The cells that eventually form the sternum, arise in the lateral walls of the thorax and then migrate ventrally towards the mid-line. Here the opposing streams meet and turn still further ventrally so as to build the keel of the sternum. Once the cells are formed in the required locality their migration becomes an inherent property of the developing tissue; isolated portions of chest wall explanted *in vitro* show almost unidirectional migration of the cells (Chen, 1952; Fell, 1939), instead of the more usual radial pattern or the "to and fro" movements seen when cells are forced by external factors into parallel array (see p. 12). Not all the cells involved in such formative movements are eventually used in the construction of the tissue, and it is not uncommon to find, in this example and others of similar kind, that many of the cells degenerate and disappear. There is, at present, very little information as to which cells survive and which degenerate, but as already noted (see p. 224), whole classes of cells may thus be eliminated from what was once a mixture of cell types. To unravel this problem it will be necessary to have far better cytochemical methods for diagnosing cell families, genera, and species than are at present available.

However, the main purpose of discussing these movements at this point was to show that the onset of such movements occurs spontaneously in certain tissues, and these tissues may show the tendency to produce their characteristic movements under a variety of conditions. In the above example, the orientation and direction of movement was not disturbed by placing the tissues in tissue culture ; the cells continued to stream in the same direction, with respect to the tissue itself, as they would have followed in the body. Possibly the orientation of the cells is fixed by the arrangement of fibrils or the molecular orientation of any "ground substance" which may be present in the tissue fragment. Weiss has repeatedly shown the enormous influence such factors may have in orientating cells, and this has been very prettily illustrated by using the orientated structures of fish scales (Weiss, 1958) as a groundwork for directing the activity of fibroblasts in tissue (see p. 12). Such orientation of movement may be determined by factors associated with the tissue as such, i.e. in the local environment of the individual cells rather than in the individual cells themselves. In Weiss's experiments the primary orientation is greatly influenced by the molecular structure of the ground substance, and it is certainly theoretically possible that chemotactic agents, or other similar factors, could subsequently attract or repel the cells along the lines of orientation. It is also theoretically possible that the cells may get into the grooves with their intrinsic polarities irreversibly fixed and with their directions of movement already determined. In such an example the timing of chemotactic and orientating influences must clearly be critical. Once again, it is very important, in considering the behaviour of a tissue, to distinguish between the inherent behaviour of the cells when isolated and their behaviour in their own little local environment with the extraordinarily intricate molecular and macromolecular patterns which must develop in even the simplest of multicellular systems. These patterns may naturally be produced by the cells themselves so that any arrangement of cells involving more than one or two cells is almost bound to have within it the seeds of some organization. Each cell modifies its immediate environment and creates a special set of conditions for its next-door neighbour, which in turn can alter the conditions further for the first cell, so that groups of cells necessarily tend to behave differently from single cells, and the original cellular constitution of the groups may rapidly lead to tissue specialization of a unique kind. Thus, once a tissue, however simple, has developed, there is likely to arise, by virtue of its own internal organization, a certain propensity for its cells to migrate in a particular manner characteristic of that tissue. This propensity must also tend to persist until something alters it.

In other words, as soon as an embryo tends to build up a certain pattern, it immediately creates, for its constituent cells, local environments, which then may themselves determine the subsequent patterns of behaviour and distribution adopted by the cells. In this way it may appear as if each step in the attainment of the final pattern was inherited; and the final pattern may persist quite independently of whether it continues to serve any useful function. In some cases a pattern may have started and been selected initially as an adaptation to some local conditions, and thus it may have a hereditary foundation which leads to the formation of the tissue up to a certain point, but after that, and in the absence of any influence to the contrary, the development is consequential on the cell environments produced. In other words, that part of the pattern actually depending directly on genes may be several stages back in embryological development. It is interesting in this connexion that cartilage cells, isolated by trypsin digestion of limb-cartilage rudiments and subsequently allowed to re-aggregate and differentiate *in vitro*, will reform cartilage with a pattern which is recognizably different from that built by similar cells isolated from scleral mesenchyme of the same embryo and treated in the same way. The cells are essentially mechanocytes in each case but belong, as it were, to two different species and their behaviour is type specific (Weiss and Moscona, 1958). The field of embryology is also strewn with examples of characteristic patterns of cells and tissues which were originally caused by formative movements of cells but which now no longer proceed to completion in the original manner but are diverted towards other functions: the islets of Langerhans in the pancreas afford an example. Thus, cords of cells frequently occur, as, in this example, between the islets and the ducts, where perhaps the ancestor had tubules, and where indeed tubules may still develop in closely related species. Once the system for the formation of tubules by the "invagination" and alignment of a particular group of cells has been established, it is probably the local environment of those cells which is actually determining the tubule-formation, and, if the pattern of development in the next generation repeats itself up to this point, tubule formation is likely again to result, whether or not the tubules are later going to be used. To understand the meaning of any such cords and patterns it is obviously necessary to study the particular tissue not only as it changes embryologically, but also to see how it is represented in comparable species in order to obtain some idea of its phylogenetic development. As another classical example, the gill slits of the mammalian embryo may be cited. They develop as in more primitive animals but they never function as gills, and secondary structures now develop from what were once functional respiratory passages, but these secondary structures depend for their particular development on the precise pattern initially set up by the formation of gill-slits. The whole process of the formation of the gill-arch is a modification of pre-existing patterns. Nevertheless, some of the original cell movements necessary to gill-arch formation still go on and, because they go on, the structures which develop can only be re-adapted to new purposes under the changed conditions. At this stage Nature can only build new structures with the materials available.

In all embryological processes leading to the formation of local environments for cells and of local "self-differentiating" systems, the time-scale of operations may be very important. For example, in the formation of some organs, two essentially different tissues may combine as in the adrenal gland, the pituitary, the eye, urinogenital ducts, teeth, and many others. The cells of each approach those of the other and eventually they unite together to form the composite organ. Each tissue then probably influences the other and complex special environments develop for the individual cells. In any one species, tissue A may approach tissue B and so appear to determine the further differentiation of B; this, however, may be a completely wrong interpretation. In the primitive condition, B may have developed first and approached A, but in evolutionary history the development of A, being independent of B at this stage, may have been accelerated for some quite different reasons, and have got ahead of B and now appear to be the determinant. It is therefore very dangerous in embryology to decide such problems on the embryological data alone, without the help of a very wide phylogenetic study of the situation. Again, certain cells might tend to leave an epithelium at a given stage of its development in order to form a gland or tube, and the actual gland or tube formation by these cells might subsequently be dependent on a proper organization of the under-lying fibroblastic tissue as has already been discussed. It would obviously be possible for the critical change to occur among the epithelial cells before the fibroblastic tissue had reached the necessary organization, so that the cells might well leave the surface and burrow into the mesenchyme, as before, but they might not develop actual tubular or glandular structures until a much later stage, i.e. when the fibroblasts had caught up. Meanwhile the cells from the epithelium might remain inconspicuously among the mesenchyme cells. Such a situation would easily give the impression that the tube might be formed by epithelial invagination in the first case, and directly from mesenchyme cells in the second. In actual fact it would be epithelial in both cases, but in the second case the epithelial cells, because they lacked the typical tubular organization which is dependent on fibroblastic influence, might well escape notice and be confused with the mesenchyme cells. Something of this sort may well account for the apparent vagaries in the formation of the archinephric duct in different species of vertebrates, as will be discussed later.

It is thus obviously a matter of the greatest importance to the understanding of the behaviour of the tissues in the higher organisms to know, in each case, exactly what cells have been involved in their make-up, and how these cells have come to assume the functions they now perform, for this latter process almost certainly depends very largely upon their previous history—including of course their phylogenetic history as well as, and in fact probably rather more than, their ontogenetic history.

In the chapters that follow, the differentiation of certain special groups of tissues will be discussed from the point of view which has been developed in the preceding pages.

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CHAPTER 13

COELOMODUCTS, NEPHRIDIA AND KIDNEYS

IN an earlier chapter it has been suggested that the primitive colonial organism may have been something very like the early blastula of a calcareous sponge of the present day. In other words, it was likely to have been a hollow sphere of cells which acquired osmotic and ionic stability by virtue of the fact that it was composed of two sorts of cells, one corresponding to the flagellate form of *Naegleria* and adapted to low ionic concentrations and the other corresponding to the amoeboid form of *Naegleria* and adapted to higher ionic concentrations. While this basic pattern of two distinct cell types is the simplest concept, it was also shown earlier that the two types should more properly be regarded as two extremes of cellular activity, that cells with intermediate properties and even reversible behaviour were probable in such organisms and that there was indeed evidence for their existence in those sponges which show cells transitional between the flagellated cells and the archaeocytes.

Exactly how these cells regulate the ionic and water balance of the organism remains to be determined. The actions of cells living as isolated units, in an unlimited environment as do the free-living amoebae, may assume a very different form when the cells become orientated as a barrier separating a blastocoele cavity from the external world, especially since survival of the whole may depend on the ability of the cells to maintain the stability of the organism and of its ionic content. In the long run, this stability is likely to be the ultimate determining factor in setting the level of activity for the two cell types, and some features of the concentrations of the various cations (including H^+) and anions (including OH^-) in the blastula cavity is likely to be the operative stimulus. This stimulus, however, acts only on one pole of the cell, while the other is being simultaneously presented with a set of conditions which may be very different. The information available from the responses of *Naegleria* to changed ionic conditions shows only that cells do respond to such changes of ionic concentration, but it does not show how they would respond to two different conditions simultaneously presented to the two ends of the cell. That cells must be able to act differently at opposite poles is self-evident from the study of any secretory epithelium, and it is interesting to notice, for example, that when the cells of sponges are dissociated, the choanocytes are flagellate at one end and actively amoeboid at the other, as *Naegleria* is during the transitional period. Similar observations have been made by Holtfreter (1947) on ciliated cells isolated from amphibian embryos (see Fig. 7.1).

At this stage, therefore, it is fair to assume that movements of ions and water can be performed by the covering cells and that the two types of cells are likely to work together for their mutual benefit. It is also fair to assume that, although the external medium may modify their activity temporarily, in the long run, the activity of both types is likely to depend primarily on the composition of the blastocoele fluid because it is this fluid which ultimately means stability and this stability carries with it the further possibility of using the cavity as a suitably protective environment for reproductive cells and the like.

SOME CONSEQUENCES OF INCREASE IN SIZE OF THE SIMPLE METAZOON

In the early stages of the development of the colonial organism no special excretory system, apart from these regulatory mechanisms, was necessary. Each cell could carry out its own excretion directly and at the same time contribute to the ionic balance of the whole. Excretion of noxious metabolites to the outside of the organism was presumably effected by both types of cell.

In the next stage of evolutionary development, however, more and more cells, as we have seen, left the surface of the organism, entered the blastocoele cavity and there lived in the regulated and mucoprotein containing fluid, which filled the interior. These cells were thus almost completely shut off from the external world and enjoyed a considerable degree of homoiostasis, but at the same time became entirely dependent on the cells of the outer shell for their nutritional requirements. (cf. p. 42).

So long as only a few of these cells were present they would make very little difference to the general stability of the organism and they would be able to live a normal, if restricted and dependent, existence. With increase in their numbers, however, conditions would inevitably tend towards the insanitary and, for the further survival and development of the organism as a whole, special mechanisms would become necessary to prevent the necrosis of the more deeply buried cells. Moreover, those particular cells from among the amoebocytes or archaeocytes, which were so segregated into the protected environment of the blastocoele cavity for the purpose of keeping them secure until they were needed as germ cells for the reproduction of the species, would need to have well-regulated conditions; they would need adequate food and oxygen and efficient mechanisms for the elimination of CO_2 and other waste products.

An examination of the organization of the Platyhelminthes reveals some very interesting and relevant facts, for these are animals existing at the present time at very much this stage of organization, i.e. with a surface epithelium and an inner mass of cells. Platyhelminthes are essentially organisms consisting of a ciliated epithelial surface, a gut cavity, whose epithelium is, of course, also a part of the external surface though the cells are not always ciliated, and an inner mass of cells almost completely filling the cavity between the two layers (Fig. 13.1).

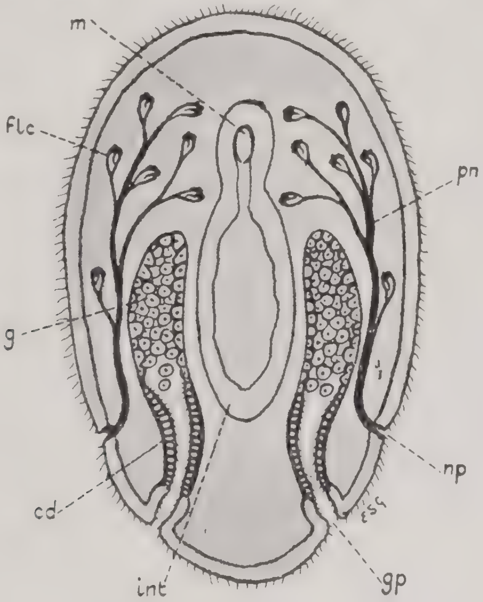

Fig. 13.1.

Plan of a primitive Platyhelminth. flc. flame-cell; g. gonad; gp. genital pore; int. intestine; m. mouth; np. nephridiopore; pn. protonephridium. (Goodrich, 1945).

In this inner mass two new systems of cells have developed. The first is a system of cavities into which the germ cells are segregated. These cavities later become connected by tubes to the outside surface. This is the first sign of a coelomic cavity and of the development of a coelomoduct connecting the cavity with the outside. The other system is a tubular system composed of cells of the external surface which enter the inner mass by pushing inwards from the external epithelium. These tubes end blindly in "flame-cells" or solenocytes and are excretory nephridia (Figs. 13.1, 2 and 3). While the "coelomic" cells may be somewhat modified from the more primitive surface condition by virtue of their enclosure, the cells of the nephridial tube need not be assumed to be very different from those of the primary epithelium, except in so far as they have acquired special activities in connexion with their continued regulatory function which now goes on between the interior of the animal and the lumen of a narrow tortuous tube instead of the unlimited external environment.

An important function for the cells of both these systems is to regulate the composition and ionic content, in the first case, of the fluid within the cavity and bathing the germ cells, i.e. the coelomic fluid, and in the second case, of the general "tissue fluid", i.e. what is left of the original blastocoele fluid. The cells of the two systems are thus oppositely polarized with respect to the blastocoele fluid or inner mass.

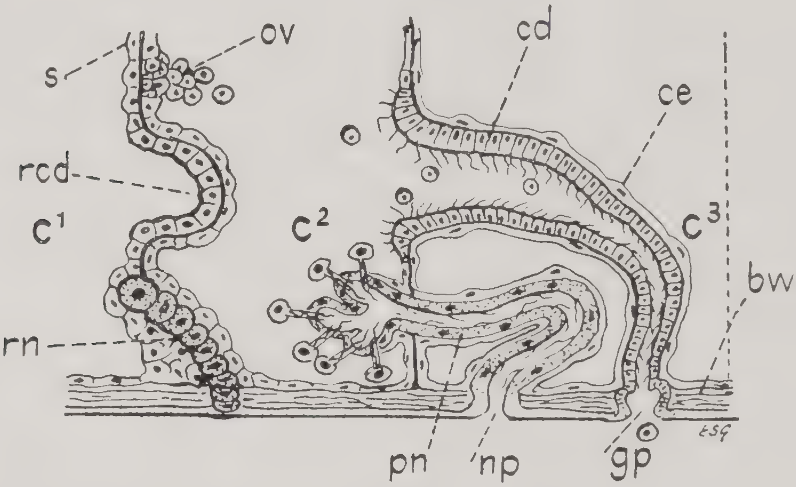

FIG. 13.2

Diagram showing the relationship between coelomoducts and nephridia in an Annelid. C^1, C^2, C^3, three successive coelomic cavities. Developmental stage on left. Adult stage on right. bw, body wall; cd, coelomoduct; ce, coelomic epithelium; gp, genital pore; np, nephridiopore; ov, ovary; pn, protonephridium with solenocytes; rcd, rudiment of coelomoduct; rn, rudiment of nephridium. (Goodrich, 1945).

NEPHRIDIA

The function of the flame cell of the nephridium is to pass fluid, drawn from the parenchyma and probably containing excretory products, into the tube and so to the outside. In the terrestrial nemertine worm, *Geonemertes dendyi*, where a very similar protonephridium is found (Pantin, 1947), (Fig. 13-3), the flame cells have been shown to function in this way and to grade their activity in relation to the water available to them: the activity of the "flames", which are really clusters of flagella, increases with the water content and the degree of stirring of the tissue fluids in the immediate vicinity of the flame cells. A similar effect of increased water content on the beat of cilia has also been observed in the ciliated cells in the nephridia of the more highly organized annelids, *Lumbricus terrestris* and *Allolobophora chlorotica* (Roots, 1955).

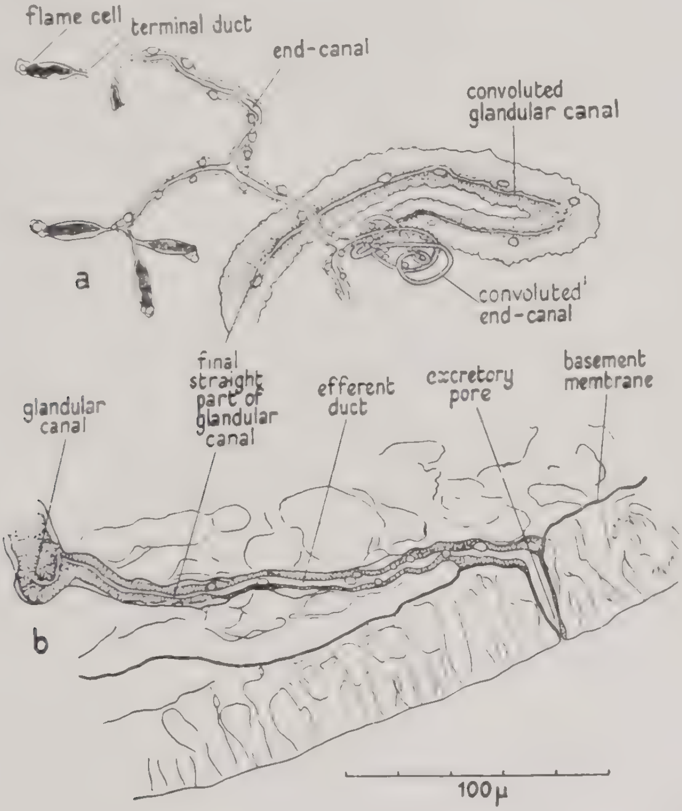

Fig. 13.3
The nephridium of *Geonemertes dendyi.* (Pantin, 1947).

The rhabdocoele, *Gyratrix hermaphroditus*, may live in either fresh or salt water, and the protonephridia differ in the two situations (Fig. 13.4). In the forms taken from fresh water, the nephridial tubes have along their walls large granular cells, called paranephrocytes, and these cells do not occur in the forms which are taken from brackish or sea water (Kromhout, 1943). It is tempting to believe that these latter cells are concerned in some way with salt conservation or with water ejection so that the fresh-water form can maintain the necessary ionic balance of its tissues. A similar difference in the structure of the nephridia exists in two species of polychaetes, *Nereis diversicolor* and

Nereis cultrifera which are closely related to each other but have different habitats (Ellis, 1939; Jurgens, 1935). *Nereis diversicolor* adapts easily to changes in salt concentration in the water and has a complex nephridium while *cultrifera* is not so adaptable to salinity changes and has a simple nephridium (Fig. 13.5).

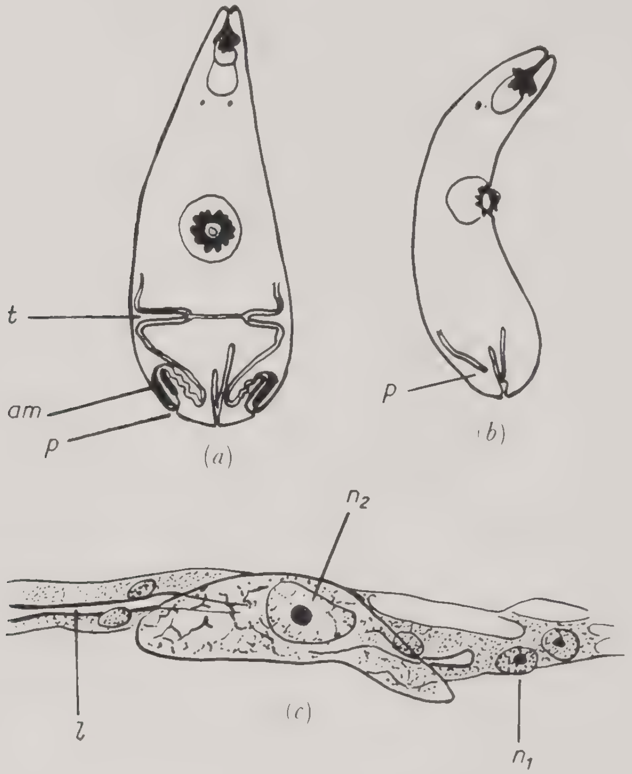

FIG. 13.4

Nephridia of *Gyratrix hermaphroditus* (*a*) Animal taken from fresh water; (*b*) Animal taken from salt water; (*c*) Paranephrocyte in the wall of the nephridial tube from a fresh-water specimen. am, ampulla; l, lumen of nephridial canal; n_1, nucleus of tubule cell; n_2, nucleus of paranephrocyte; p, pore of nephridium; t, nephridial tube with transverse canal. (Redrawn from Kromhout, 1943).

The nephridial tube can thus probably be looked upon as a special tubular invagination of the superficial epithelium containing, potentially at least, cells of both forms, and the main function of these tubes is presumably the preservation of the ionic balance of the inner mass, and, as seen in *Geonemertes*, their activity is dictated by that ionic balance. In relation to the inner mass, the flagellate cell must be regarded as using its inner membrane as a water collector (either active or passive), and the cell then ejects the water possibly with contained salts, at the flagellate end. The paranephrocyte on the other hand could well be absorbing salt from the tubular fluid and ejecting it across its inner membrane into the intercellular fluid according to the concentration in that fluid. The absorption of water may also be a very necessary property of the nephridial tubes of many terrestrial or marine animals. It is possible to imagine that the flagellum itself may be the means of transport of either water or ions by virtue of the contractile proteins which it contains. This being so, a further complication is raised and should certainly be borne in mind. In Protozoa, flagella may either act as tractella or pulsatella according to the point of origin of the wave and the direction of its travel. *Naegleria* has tractella, so if it is supposed, for example, that this constitutes the Na^+ collecting mechanism, then a flagellate cell with pulsatella could conceivably be a Na^+ ejector. It may therefore not be sufficient to classify cells, as we have done, simply on whether or not they have flagella (or cilia); it may be necessary to study the matter further in relation to the direction of beat. For example, certain nephridia have flame cells which pass fluid into cellular tubes which also have long flagella in the lumen. The flame cells contain no alkaline phosphatase, but the flagellate tube gives a strong positive reaction for it (Danielli and Pantin, 1950), so that in this respect at least the cells are clearly rather different in kind. Is this perhaps a case of the flame cells having one type of flagellar motion, and the tubule cells having the opposite? The reversal of ciliates and flagellates on coming into contact with acid media or changes in salt concentration perhaps should also be considered from this point of view.

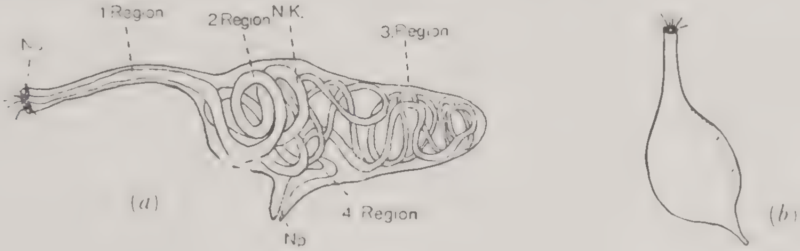

FIG. 13.5

(a) Nephridium of *Nereis diversicolor*. Ns, nephridiostome; Np, nephridiopore; Nk, nephridial canal. (b) Nephridium of *Nereis cultrifera*. (Jurgens, 1935.)

Because an animal has developed these nephridial tubes it does not necessarily follow that all excretion and water balance must take place through them. The outer surface may still, in fact, perform the lion's share of the work. If the estuarine worm, *Gunda ulvae*, for example, is placed in fresh water containing calcium, some degree of equilibrium is maintained (Pantin, 1931) as an active process requiring energy, but the gut cells become extremely vacuolated (Beadle, 1934) and seem to be the chief cells which show change of function in the two media (Fig. 13.6). Moreover, if the worm is poisoned with cyanide the gut cells remain more nearly normal in appearance and the parenchyma swells with fluid. This strongly suggests that the gut cells are playing a leading part in the maintenance of water balance, but it does not say that the nephridial tubes are remaining entirely inactive in the transfer of either water or ions, though they are not visibly changed when the osmotic pressure of the surroundings is changed (Beadle, 1934). Interestingly enough, water does not appear to be discharged from the vacuoles of the gut cells to the outside, which perhaps suggests that they may be tending to prevent the dilution of the tissue fluid by ejecting salts into the parenchyma, or at any rate that they are not doing it in the manner in which the flame cells of *Geonemertes* do it, i.e. by passing fluid directly to the outside.

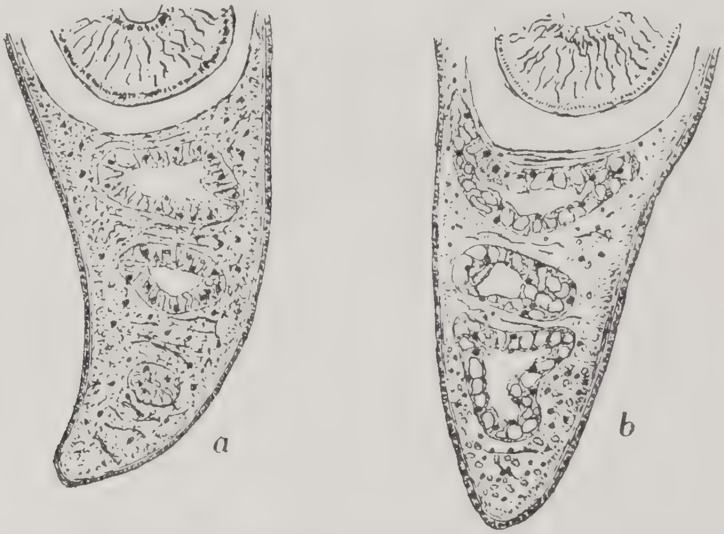

FIG. 13.6

Sections of the flatworm *Gunda ulvae* (a) from 100% sea-water (b) from 10% sea-water: Note the vacuolation of the intestinal cells. (Beadle, 1934).

In *Geonemertes* (see Fig. 13.3), the nephridial tube has a pattern of considerable interest in that it seems to foreshadow the structure of excretory tubes in a very wide range of more highly organized animals. The tube is headed by a flame cell and, as already described, this leads into a rather thin-walled ciliated tube which, after some convolution, opens into a thick-walled "glandular" canal. This then gives place to a thin-walled duct leading to the excretory pore. In some ways it is rather like a blastula but turned inside out—flagellate cells at one end and "glandular" cells at the other—and this resemblance is probably not just fortuitous; the "glandular cell" may, indeed, be the modified version of the posterior cells of the blastula. The nephridial tube is thus doing exactly the same for the inner mass of cells as the surface cells do for those parts of the inner mass within their reach, and the activity of the cells remains under the control of the ionic content of the original "blastula" fluid as it impinges on the basal cell membranes of the surface cells. The nephridial cells also are primarily orientated to regulate the tissue fluid: the contents of the nephridial tube are of as little importance as such to the animal as the general surrounding fluid. It is not suggested that ionic regulation is the only activity of the nephridial cells, any more than it is of the surface cells, but in both cases it must loom large in the general determination of activity.

COELOMODUCTS

The cells lining the coelomic cavity are, as already suggested, presumably responsible for the secretion and regulation of the fluid in the coelomic space which they form and then surround. Either all the cells are totipotent, and can therefore develop in any direction or, as seems to be more likely, they contain among their number some cells which remain totipotent because it is from among the cells of the coelomic epithelium that the definitive germ cells emerge. The cavity probably develops initially within the mesohyl and only secondarily opens to the exterior: it develops for the purpose of providing a space with a constant environment for the germ cells, where these cells may be protected from the majority of external influences and so have the continuity of their hereditary constituents assured; it provides, as it were, the fourth step in the protection of the deoxyribosenucleoproteins (genes), the preceding steps being (1) protection within cytoplasm; (2) protection within the nucleus; and (3) protection of the whole germ-cell within a blastula-like organism. The cells lining the coelomic space, other than the germ cells themselves, are thus the cells which are immediately concerned with the coelomic fluid. The composition of the fluid in the actual coelomoduct as distinct from the coelomic cavity, may be of little consequence to the organism at this stage, except that valuable metabolites must not be lost to the organism, but the production and the composition of the fluid in the main cavity itself is important. The two original cell types present in the mesohyl, but perhaps with some modification, are again available for regulation of the coelomic fluid and, when coelomoducts develop by outgrowth of the coelomic wall to the exterior, for the regulation of the fluid in the coelomoducts themselves. In the coelomic wall, however, the stimulus for modification of activity is more likely to be the fluid in the cavity or duct acting on the apical cell membrane than, as in the case of the surface epithelial cells and nephridia, the tissue fluid of the mesohyl acting on the basal cell membrane. In other words, the coelomic cells have, as it were, reversed their polarity with respect to the mesohyl, but their functions are closely similar in many other ways, to those of the nephridial cells: each system is regulating a fluid, but the fluids are different.

The Relationship between Nephridia and Coelomoducts

As the coelomic cavity increased in size and importance in the more highly organized animals, an early modification of the original pattern of two entirely separately developed regulatory systems emerged. The nephridial solenocytes projected into, and drew their fluid from the coelomic cavity rather than from the tissue fluid; and the coelomic fluid as such became an important integrating fluid of the body, and the one most in need of regulation. So much was this the case that the next step seems to have been that the nephridia actually opened into the coelomic cavity by means of a ciliated funnel and drew coelomic fluid directly into the nephridial canal. At this stage the regulatory functions of coelomoduct and nephridium became almost identical, namely, the regulation of coelomic fluid and tissue fluid and the prevention of the loss of valuable metabolites to the outside world through the nephridial canal. This combined system would have the advantage of having essentially four sets of cells capable of contributing to it and of co-ordinating their activities, two sets taking their cue from the composition of the coelomic fluid, the other two from the tissue fluid. It is therefore not surprising that with the varying relative importance of coelomic and tissue fluids in different animals, all sorts of combinations between nephridial elements and coelomic cell elements have occurred (see Goodrich, 1945) (Fig. 13.7). Among the various phyla of animals, and particularly among the annelids, coelomoducts open into nephridia making all manner of junctions in different groups, but, interestingly enough, nephridia do not often seem to open into coelomoducts. The sequence is always coelomoduct to nephridium, except perhaps in the Acanthocephala (Goodrich, 1945). Does the reason for this lie in the point just made, namely, that the composition of the fluid finally excreted from the organism is of little consequence to the organism, but the composition of its tissue fluid is vital, so that the last word should be said by nephridial cells and not by those of the coelomoducts?

There is not much information about the composition of the coelomic fluid in the very primitive organisms, but for certain earthworms (*Pheretima posthuma*) with well-developed coelomic cavities, coelomoducts, and nephridia a few figures are available (Bahl, 1945,

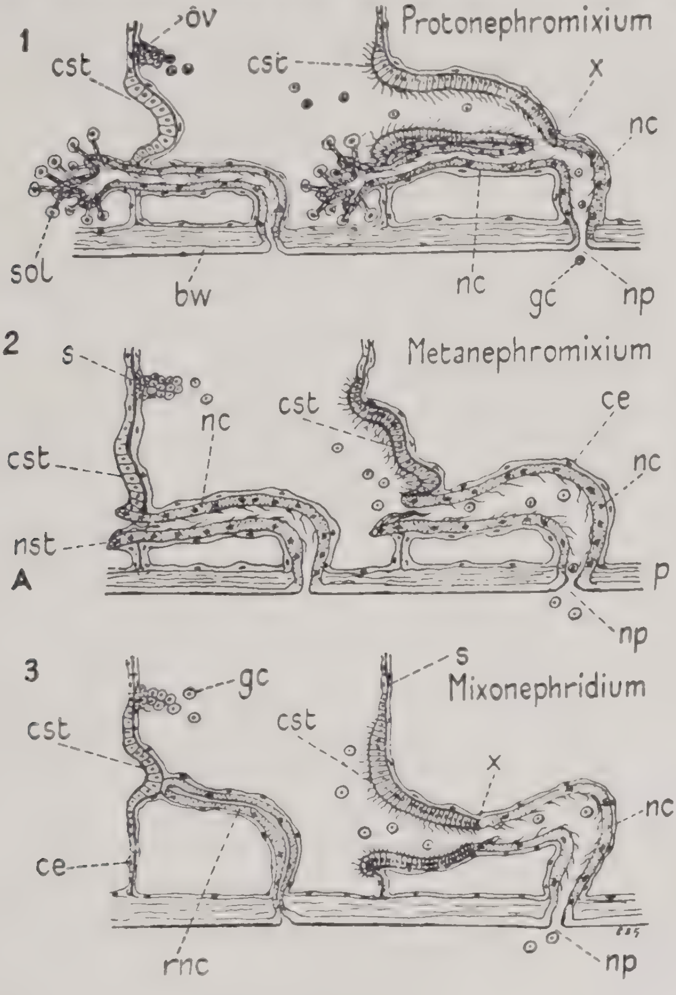

FIG. 13.7

Diagrams showing structure and formation of nephromixia by combination of coelomoduct with nephridium. A. Anterior, young stage before combination. P. Posterior, combination completed. bw, body wall; ce, coelomic epithelium; cst, coelomostome; gc, germ-cell; nc, nephridial canal; np, nephridiopore; nst, nephridiostome; ov, ovary; rnc, rudiment of nephridium; s, intersegmental septum; sol, solenocyte; x, point of junction. (Goodrich, 1945).

1947), so that some sort of picture may be obtained of how the mechanism can work (Table 13.1). No glucose is found in the coelomic fluid of these worms and there is much less protein than is found in the blood. On the other hand, sodium and chloride are more concentrated, and potassium and phosphate less concentrated than in the blood. Unless all the glucose is used by the surrounding cells or the corpuscles in the cavity, which seems extremely unlikely, its absence suggests that glucose must either not be secreted into the fluid or must be actively absorbed back into the blood or tissue fluid by the cells lining the coelomic cavities. It is unlikely that the nephridia could play any part in this activity by reason of their position with respect to the bulk of the coelomic fluid. A transudation of fluid into the cavities combined with a reabsorption of glucose would explain the findings most simply, provided that enough fluid would move into the cavity. The absence of protein from, and the very low sodium content of, the urine suggest active reabsorption of these substances by the nephridium.

These figures for earthworms are interesting in themselves and illustrative of the manner in which the cell types with which this chapter is concerned may work, but it must be emphasized that annelids are specialized creatures with their own requirements, so that the way in which their cells have evolved may differ both qualitatively and quantitatively from the line taken by the corresponding cells on the vertebrate and mammalian line of descent. The available figures for the coelomic fluids of elasmobranch fishes indicate a remarkable degree of variability in the composition of these fluids (Smith, 1929). The pericardial fluid of *Raia* is more acid than the blood, contains more

TABLE 13.1

Constituent	*Earthworm's blood* (*g.*/100 *c.c.*)	*Coelomic fluid* (*g.*/100 *c.c.*)	*Earthworm's urine* (*g.*/100 *c.c.*)
Water	89·76	98·87	99·12
Glucose	0·10	Nil	Nil
Proteins	3·64	0·48	0·030
Amino acids	0·006	Nil	0·00004
Fats	0·20	Nil	Nil
Ammonia	0·004	0·0027	0·0027
Urea	0·0026	0·0025	0·0032
Uric acid	Nil	Nil	Nil
Creatinine	0·0035	0·0027	0·0005
Na	0·095	0·185	0·0235
K	0·0738	0·0231	0·0092
Ca	0·017	0·0225	0·012
Mg	0·007	0·0397	0·0054
Cl	0·05	0·08	0·0037
PO_4 (inorganic)	0·0174	0·0020	0·0011
SO_4 (inorganic)	0·0033	0·0023	0·0016

Na^+ and much more K^+, but less Ca^{++} and less CO_2 than the blood plasma; it is also almost free of protein. The peritoneal fluid, on the other hand, is also acid and somewhat lacking in Ca^{++} but is very rich in Mg^{++}; its Na^+ and K^+ are not very different from those of the blood. It is obviously difficult to draw conclusions from such figures except to say that selective secretion or absorption must be going on somewhere, in the maintenance of these differences from the blood.

It may be said that the main physiological distinction between the cells of the coelomoduct system and those of the nephridial system is the method by which the orientation of their activities, either away from the tube into the tissue spaces, or *vice versa*, is determined. In coelomoducts, such activity must be governed by the action of the coelomic fluid itself on the apical surface of the cell; in nephridia the ruling factor is the action of the tissue fluid on the basal surface. This primary difference may prove to be a more reliable guide to the understanding of the relationships of execretory and related cells in higher groups than, for example, the direction of growth of the tubules in embryology. In general, coelomoducts grow outwards from mesohyl to the surface, while nephridia grow inwards as epithelial invaginations, but in the oligochaetes the simple pattern of the development of the latter has been modified to such an extent as to be almost unrecognizable and this could well have happened in other animals also. The cells destined to form the nephridia are budded off separately and at an early stage into the underlying mesohyl as nephridioblasts. It is only later that the products of their division again make contact with the surface, as a cord of cells that ultimately hollows out to form a tube (Meyer, 1929) (Fig. 13.8). Such modifications of the original method by which nephridia are formed would seem to lessen the difference in kind between cells of the coelomoducts and those of the nephridia except in the matter of their polarity; both may have a period when they are enclosed in the mesohyl and both are concerned with body-fluid regulation. The nephridioblasts must presumably be regarded as "intermediate" cells which are firmly determined neither as archaeocytes nor as potential flagellate cells, for both "flame cells" and "glandular" cells develop from them. From this point of view, the development of the first larval protonephridium of the archiannelid, *Polygordius*, directly from a cell of the third quartette of the embryo is interesting in that such a cell would occupy an intermediate position in the "animal-vegetal" gradient of the embryo and thus be, theoretically at least, capable of producing cells of both main types.

As already indicated, the coelomic cavities, originally small and specialized for the germ cells only, came to occupy a much larger proportion of the animal's bulk as evolution proceeded; and the germ cells came to be restricted to more and more localized regions of the cavities. The coelomic fluid began to dominate the activities of the regulatory mechanisms, and nephridia came into close contact with, or actually opened into, the coelomic cavity (Fig. 13.9). This they do not only in annelids but to some extent also in animals as high in the evolutionary scale as the chordates, e.g. *Amphioxus* (*Branchiostoma*) and *Asymmetron* (Goodrich, 1945) (Fig. 13.10). In the nemertines, on the other hand, there are two interesting developments which seem to foreshadow future events both in the direction of the segmental worms and in the direction of the vertebrates (Fig. 13.11). In the first place, some of the nephridia come into close communication with the blood

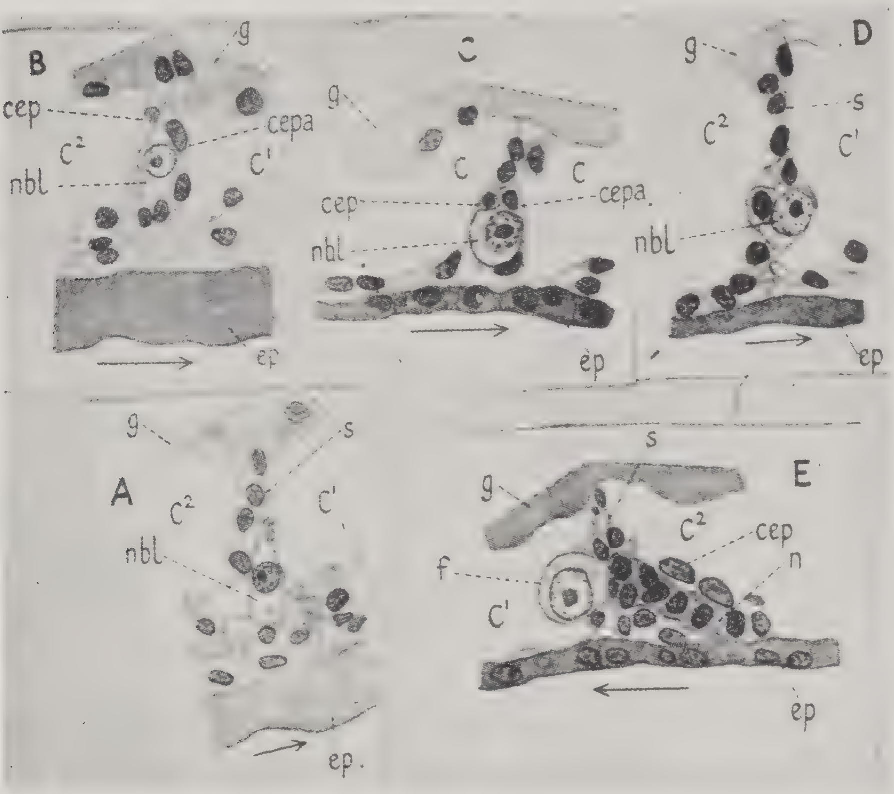

Fig. 13.8

Stages in the development of the nephridium in *Tubifex rivulorum* as seen in progressively more anterior segments of the worm. A. Septum 20/10; B, 19/18; C, 14/13; D, 13/12. E. Similar section of older stage. $C^{1\,2}$ Consecutive coelomic cavities; cep, coelomic epithelium, posterior face of septum; cepa, coelomic epithelium, anterior face of septum; ep, epidermis; f, funnel cell or residual nephridioblast; g, wall of gut; n, row of nephridial cells. (Goodrich, 1945).

channels rather than the coelomic spaces, thus suggesting that in these worms this fluid is becoming, as it obviously does in the vertebrates, an important co-ordinating fluid. It is, of course, the one whose composition eventually determines the whole physiological pattern of higher animals. The solenocytes of nemertines sometimes project into the haemocoele cavities, which are filled with blood. Another interesting feature of the nemertines which may be relevant to future arguments, is the fact that several nephridia which were originally arranged in a serial (presegmental) manner may be connected by a common duct which may have one or more openings to the exterior. This situation in the nemertines leads to some interesting speculations on the nature of the urinogenital system and its ducts as these appear in the vertebrates. They can only be speculations because the pre-vertebrate ancestors are themselves matters for speculation.

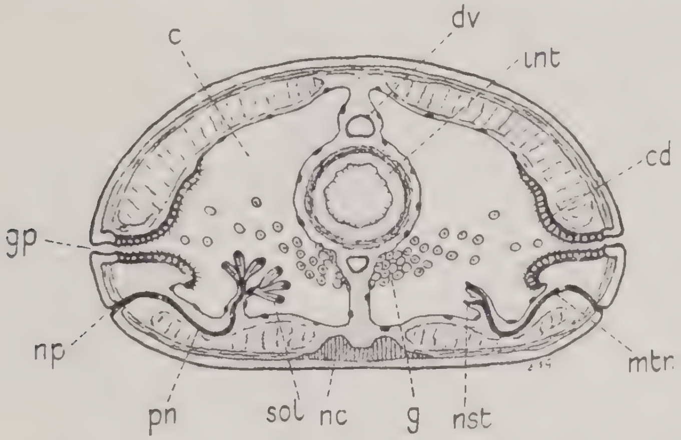

FIG. 13.9

Diagram showing two methods by which nephridia come into close contact (left) or actually open (right) into the coelomic cavity as seen in Annelids. c, coelomic cavity; cd, coelomoduct; dv, dorsal vessel; g, gonad; gp, gonopore; int, intestine; mtn, metanephridium; nc, nerve cord; np, nephridiopore; nst, nephridiostome; pn, protonephridium; sol, solenocyte. (Goodrich, 1945).

THE URINO-GENITAL SYSTEM IN VERTEBRATES AND ITS EVOLUTION

Before discussing this aspect of the problem, it will probably be helpful to outline some of the general features of the vertebrate excretory system, not so much from the aspect of the detailed cytology of the adult kidney as from the point of view of its basic structure. On both phylogenetic and ontogenetic evidence there is a strong probability that the kidney derives from serially arranged tubes, many of which opened at one end into the coelomic cavity and most of which open at the other end by a common duct to the outside (Fig. 13.12); but these tubes may well have each opened separately at one time and only united as a secondary phenomenon. The linking together of the nephridial ducts of the nemertines is a parallel case of such antero-posterior connexion of serial units. The same process has occurred also in the annelids where the nephridial tubes in the posterior segments of several species, e.g. *Hoplochaetella* and *Allolobophora* all unite to a common duct which then opens at the cloaca or into the intestine respectively (Fig. 13.13). It may be assumed, therefore, that the originally serial tubes of vertebrates have come to be replaced by a duct which gives off or receives serial branches. In the anterior region of the body these branches (constituting the pronephros) are roughly segmental and develop early. More posteriorly and separated by a zone of incomplete tubes from the pronephric units, the branches are generally concentrated into two main regions, again separated from each other by a zone in which the serial tubes may degenerate before

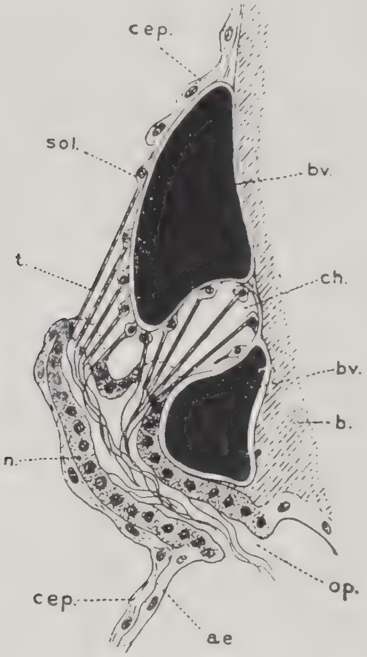

FIG. 13.10
Diagram of transverse section through the nephridium of *Amphioxus* showing the relationship between the solenocytes and the coelomic cavity on the one hand and blood vessels on the other. ae, atrial epithelium; b, top of secondary gill-bar; bv, blood vessel; cep, coelomic epithelium; ch, chamber with solenocytes; n, wall of nephridial canal; op, nephridiopore; sol, solenocyte; t, its tube with flagellum. (Goodrich, 1945).

they ever become functional. These concentrations form the mesonephros and the metanephros, and in each of them there is some degree of secondary branching which, as in the functional metanephros of mammals, may become very extensive. Again it is interesting to observe that a rather similar proliferation of nephridia has occurred in earthworms, e.g. in *Megascolex*, *Pheretima* etc. (Bahl, see Goodrich, 1945) where each segment of the worm, instead of having a single nephridium, has a very large number of very small ones, each, in these cases, opening separately on to the surface (Fig. 13.14). In many other animals, e.g. in *Priapulus* (Fig. 13.15) and even *Amphioxus* also, multiple branching of the nephridia may occur to give rise to numerous solenocyte chambers connecting with a single duct. There is no reason

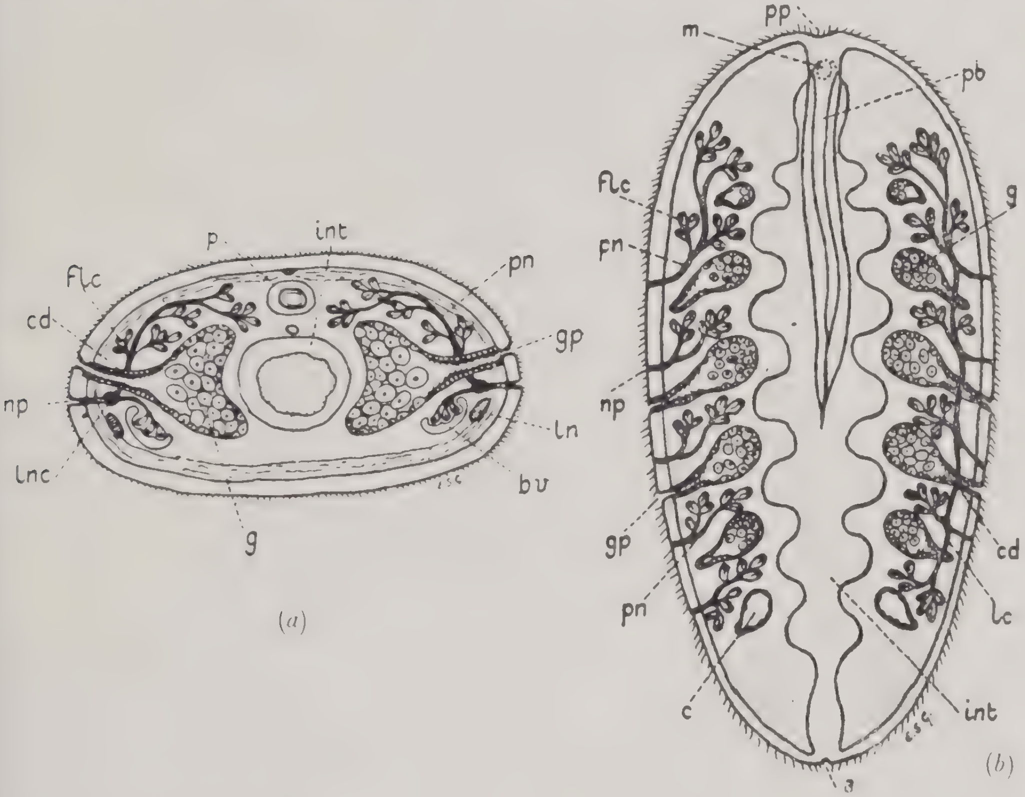

FIG. 13.11

Diagrams to show (a) the relation between nephridia and blood vessels and (b) the linking together of separate nephridia by a longitudinal duct in nemertines. (a, anus; bv, blood vessel; c, coelom; cd, coelomoduct; flc, flame-cell; g, gonad; gp, genital pore; int, intestine; ln, longitudinal nerve; lc, lnc, longitudinal nephridial canal; m, mouth; np, nephridiopore; p, pb, proboscis; pn, protonephridium; pp, proboscis-pore. (Goodrich, 1945).

to suppose therefore, that this secondary branching in any way detracts from the idea that nephrons were originally serially arranged, if not actually segmental.

There are thus three main problems in the basic organization of the excretory system of the vertebrates. First, there is the nature of the tubes themselves; are they nephridial or of coelomoduct origin, or are they more or less elaborate nephromixia, perhaps differing in different parts of the system? Second, there is the nature of the connexion which these tubes may make either with the coelom or with the blood supply or with both. Third, there is the nature of the connexion which the tubes, or certain of them, may make with the gonads or with the products of the gonads. Finally, as a subsidiary problem, some other

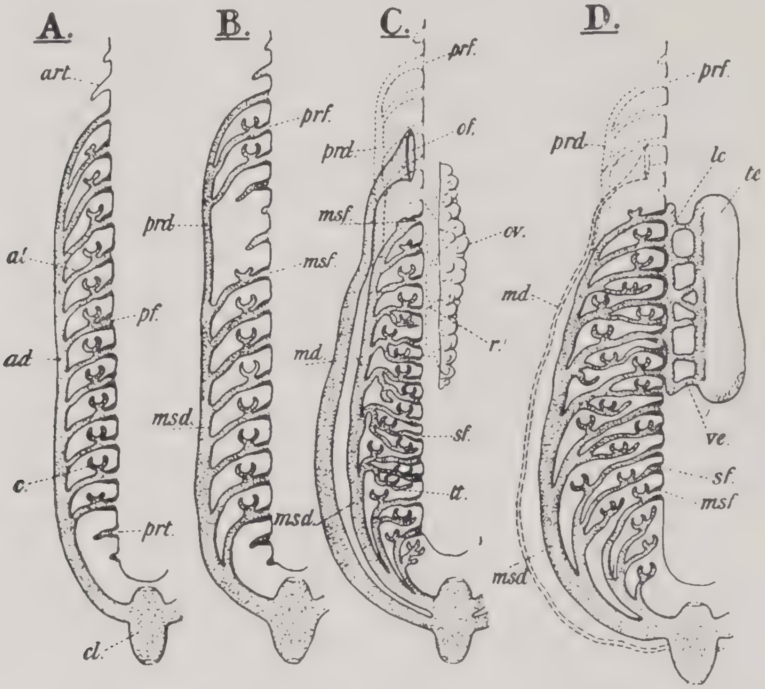

FIG. 13.12

Diagrams showing possible development of the urinogenital system in the Craniata. A. Hypothetical ancestral stage with continuous archinephros. B. Cyclostome with anterior pronephros. C. Female gnathostome. D. Male gnathostome. ad, archinephric duct; art, anterior vestigial tube; at, archinephric tube; c, Malpighian capsule; cl, cloaca; lc, longitudinal canal; md, Mullerian duct; msd, mesonephric duct; msf, mesonephric funnel; of, coelomic funnel; ov, ovary; pf, coelomostome; prd, pronephric duct; prf, pronephric funnel; prt, posterior vestigial tubule; r, vestigial network of vasa efferentia; sf, secondary funnel; te, testis; tt, tertiary tubule; ve, vas efferens. The vestigial oviduct and the embryonic pronephros are represented by dotted lines in C and D. (Goodrich, 1945).

organs may have their origin in the same series of "excretory" tubes, and a study of these organs may throw light on the main excretory system.

With regard to the nature of the tubes themselves, it may be interesting first to examine similar systems in certain other animals, which, though not necessarily on the main line of vertebrate descent, may have originated from animals closely akin to those which did lead on to the vertebrates.

Perhaps the system found in *Priapulus* is among the more interesting (see Fig. 13.15). There is a large coelomic cavity and there are paired urinogenital organs opening on each side of the anus, and running about half-way up the body. A sinus lined with columnar epithelium receives the excretory products from the rather elaborate solenocyte system on the one hand, and also receives the genital products which are conveyed to it from the serially arranged testes or ovaries by a long serially branching ciliated tube. This arrangement strongly suggests the combination of an anterior coelomoduct system with a posterior nephridial system. In this case, therefore, it is as though the coelomoducts formed a common tube; the nephridial canals also formed a common tube, and the two common tubes then united. The fact that this has happened in *Priapulus* would indicate that such things are not impossible in the vertebrates.

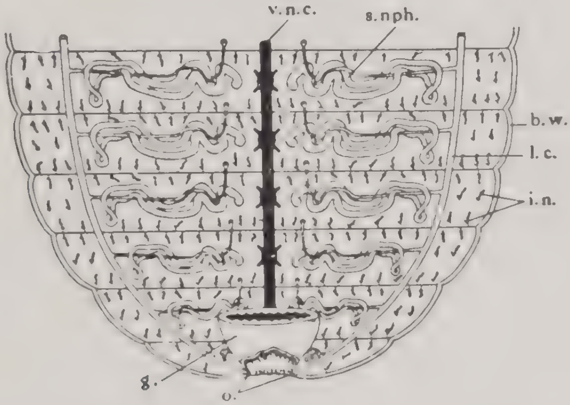

FIG. 13.13

Diagram of the nephridial system in the posterior segments of *Hoplochaetella bifoveata*. Dorsal view. bw, body wall cut open and spread out; g, hind-end of gut, the rest cut away; in, integumentary meronephridia; lc, longitudinal lateral excretory canal opening at o; snph, septal nephridium connecting with lateral canal; vnc, ventral nerve cord. (From Goodrich, 1945, after Bahl).

In the Sipunculida, the excretory organs are again nephromixia. A "yellow" cell in the mesoblast divides to give rise to three colourless cells and one "yellow" cell. The colourless cells then divide further to form a tube which opens to the coelom by a ciliated funnel which itself is derived from the peritoneal epithelium. The "yellow" cell forms the rest of the tube to the exterior. Most of the tube is thus probably nephridial in origin, deriving from the original "yellow" nephridioblast, but the coelomostome itself is probably an outgrowth of the coelomic cavity.

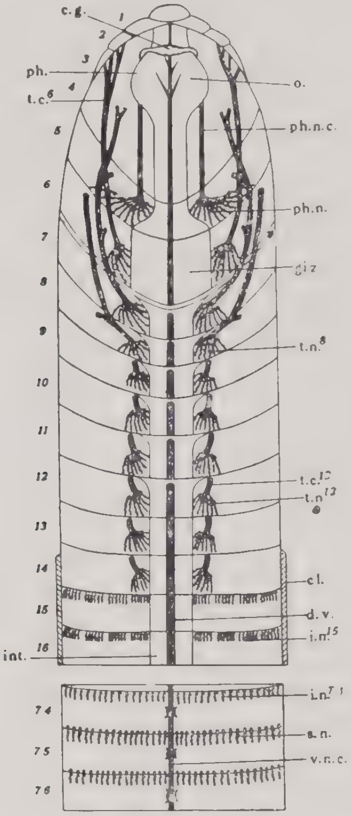

Fig. 13.14

Plan of part of the nephridial system of *Megascolex cochinensis*. c.g., cerebral ganglia; cl, clitellum; dv, dorsal vessel; giz, gizzard; in, integumentary nephridia; int, intestine; o, concealed opening of pharyngeal duct into the lumen of the pharynx, ph; phn, pharyngeal mesonephridia; phnc, bundles of ductules; sn, septal meronephridia; tc, ductules of exonephric tufted meronephridia. (From Goodrich, 1945, after Bahl).

Again, in *Phoronis* the urinogenital organs are probably coelomostomes grafted on to a nephridial canal. The young animal certainly has protonephridia with solenocytes projecting into a haemocoele. At metamorphosis these solenocytes break off and float away in the blood stream as darkly staining cells, and meanwhile a coelomostome develops from the posterior part of the coelom and opens into the original nephridial canal (Goodrich, 1903).

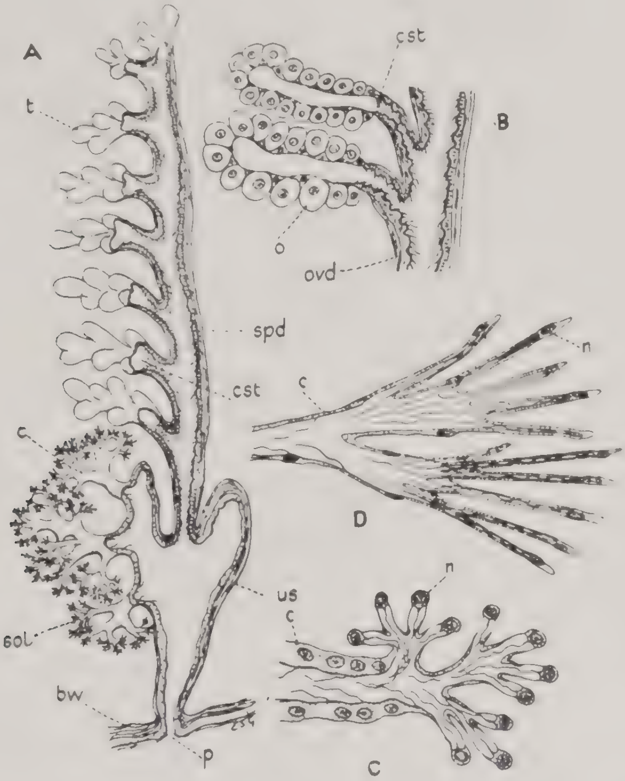

FIG. 13.15

Diagram of the urinogenital organs of *Priapulus caudatus*. A. Male. B. Part of oviduct. C. Protonephridial canal. D. Branch with elongated solenocytes. bw, body wall; c, protonephridia; cst, coelomostome; n, nucleus; o, ova; ovd, oviduct; p, urinogenital pore; sol, solenocytes; spd, sperm duct; t, testis. (Goodrich, 1945).

In *Balanoglossus* there are serial gonadal sacs, each connected by what appears to be a coelomoduct to the outside, but, in addition to these, there are the large anterior cavities, one in the proboscis and one in the collar, which are probably of coelomic origin, and which connect by one or two pores respectively to the outside and regulate the turgidity of these parts of the animal (Fig. 13.16). The nature of these ducts is obscure but there is nothing particularly labelling them as coelomostomes or as nephridia. No undoubted nephridial structures have been found in *Balanoglossus*.

In the Ascidia, and more particularly their larvae, there are no indications described of the origin or function of any excretory organs, but in *Amphioxus* and *Asymmetron* there are well-developed nephridia with solenocytes projecting into the coelomic cavity on the gill bars and opening serially on the gills. Hatschek's nephridium also appears to be a genuine nephridium and develops solenocytes (Goodrich, 1933). The mode of origin of this organ as described by Legros (1910) is almost precisely that of the pronephric funnels. In fact, this author homologizes Hatschek's nephridium with pronephric tubes elsewhere, and considers it to be an outgrowth of the nephrocoele or intermediate vesicle of the coelomic cavity of the second mesodermal segment. His views, however, do not coincide with those of Goodrich on this matter, but their implications are interesting in relation to the combination of nephridial and coelomoduct structures. The gonopores (cicatrices) which only open temporarily into the atrium may have some relationship with typical coelomoducts, but otherwise there is no sign of any combined coelomoduct-nephridial system in these creatures nor, indeed, of any purely coelomoduct system.

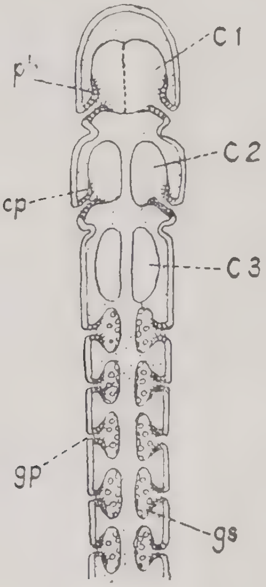

FIG. 13.16
Schematic diagram of *Balanoglossus*. Coelomic cavities, each with a coelomoduct. c^1, proboscis; c^2, collar; c^3, trunk coelom; cp, collar pore; gp, genital pore; gs, genital coelom; pt, proboscis pore. (Goodrich, 1945).

It is thus interesting to notice that in these various groups of animals which, for various reasons, are thought to have significance with regard to the origin of vertebrates, there is no uniformity in respect of their urinogenital systems; in *Priapulus* and *Phoronis* there are obvious combined excretory and genital systems (i.e. nephromixia), in which nephridial and coelomostomal rudiments are fused. In *Balanoglossus* all the accent appears to be on coelomoducts and the contribution by nephridial elements is minimal, while in *Amphioxus* nephridia are clearly present and coelomoducts are only doubtfully represented. The student of the urinogenital system in the vertebrates should therefore keep an open mind.

The cyclostomes of the present day, although mostly aberrant and in many ways specialized for their own peculiar means of livelihood, are now the most primitive animals in which the true vertebrate plan is

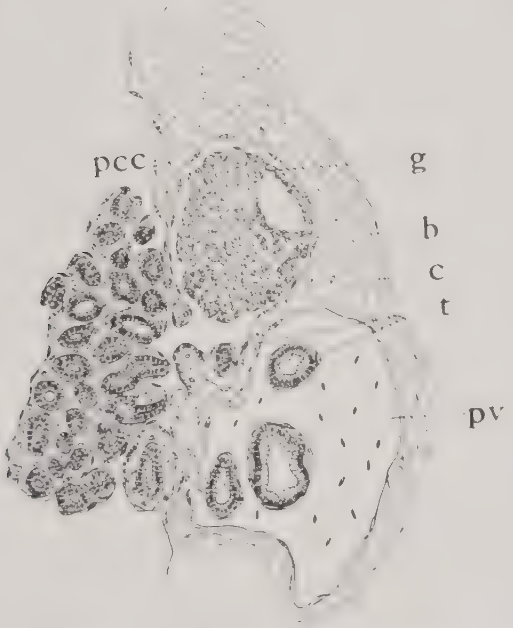

FIG. 13.17

Diagram showing arrangement of pronephric structures in *Bdellostoma stouti*. b, pocket of pericardial cavity; c, connective tissue; g, glomerulus; pcc, pericardial cavity; pv, pronephric vein; t, tubule. (Conel, 1917).

undoubtedly present; the study of their urinogenital system is therefore of vital importance. In the larval form several open coelomic funnels join together to form an archinephric duct which runs posteriorly to open at the cloaca. Near the openings of these funnels there are several knots of blood vessels (glomeruli) projecting into the coelomic cavity (Fig. 13.17). It is a moot point whether these glomeruli developed in order to provide, by filtration, a more ready flow of fluid to fill the now much enlarged coelomic spaces which have taken on the functions of body cavities for housing the main organs of the body, and thus allowing of their easy relative movement when the animal as a whole bends its body, or whether they developed near the excretory funnels in order to provide a ready escape for the excess water which tended to penetrate the tissues when the animal entered a fresh-water environment. Their developmental dependence on the presence or proximity of pronephric tissue suggests the latter. Certainly the more efficient blood system could provide the driving force for filtration and for moving the excretory fluid, and this might eliminate the necessity for active secretion of this fluid. Equally certainly there is a general correlation, in the fishes, between the development of glomeruli and a fresh-water environment (Marshall and Smith, 1930) though there are exceptions. Glucose, however, would escape through the glomeruli and would necessitate some reabsorptive mechanism in the excretory tube if this important metabolite were to be preserved. As was mentioned in connexion with the coelomic fluid of earthworms, either the coelomic epithelium or the coelomostomes have the capacity for this selective treatment of glucose, because there is none in the coelomic fluid, whereas other constituents of the blood are represented in the coelomic fluid.

The key problem therefore, is the nature of the coelomic funnels and of the archinephric duct. In recent years, largely because of the very strong evidence provided by the amphibia, it has been the fashion to regard the archinephric duct as the backward prolongation of the three or four coelomic pockets (nephrocoeles) that constitute the rudiment of the pronephros; and to consider the whole system as thus essentially mesodermal and coelomic in origin. This is certainly the simplest and easiest interpretation of most of the embryological data. There is, however, a considerable amount of evidence both from the elasmobranchs and from mammals (see Field, 1891), and most recently that of Towers (1958), who has reviewed the whole position and has himself followed the development of the archinephric duct in the sheep and rabbit, that the duct itself is phylogenetically ectodermal in origin and is progressively nipped off or "dehisced" from the epithelial surface at earlier stages in ontogeny. This ectodermal origin suggests a parallel with nephridia, and the pronephros now seems more likely to emerge as a system of nephromixia, i.e., true coelomic funnels leading to a nephridial duct in the manner originally suggested by Graham Kerr (1919) and depicted in Fig. 13.18.

Even in amphibia it is interesting to note that Fales (1935) has shown that the pronephric tubules, of which there are proximal and distal segments with brush border and rodded basal cytoplasm respectively, have a different origin from, and may behave independently both of the nephrostomes themselves (Howland, 1921) and also of the pronephric duct. The latter arises in somites 5 and 6, while the tubules arise near somites 3 and 4 and the cells are of different histological character from those of the nephric tubules themselves. What is perhaps even more significant, is that in the earliest stages of the development of the pronephros in *Rana* and *Bufo* (Field, 1891) there is an accumulation of cells between the outer coelomic layer and the ectoderm (Fig. 13.19). The origin and fate of these cells has not yet been made clear, but the resemblance between them and the nephridioblasts that appear in the sub-epidermal tissues of worms, is certainly worthy of notice. They could conceivably be cells set apart at an early stage in the same way as are the nephridioblasts of annelids, and could thus be thought of as constituting the nephridial rudiment to be joined later by the coelomoduct, of which the pronephric funnels are certainly a part.

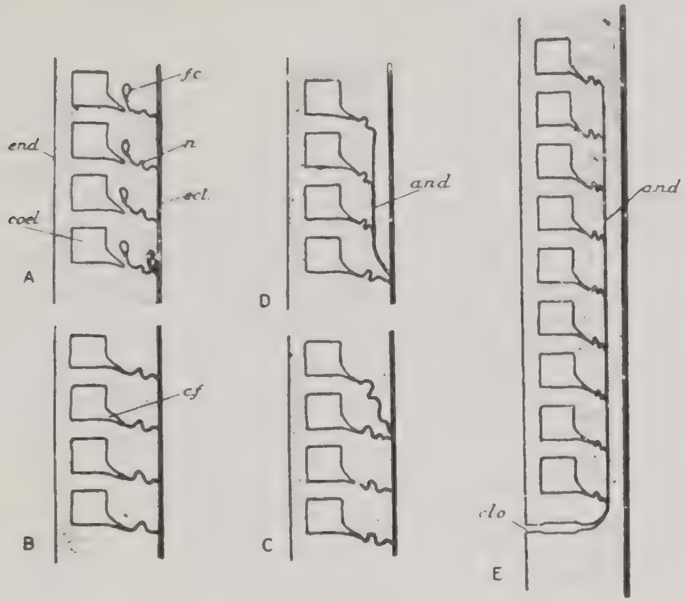

FIG. 13.18

Diagram illustrating possible stages (A to E) in the evolution of the archinephric duct. a.n.d., archinephric duct; cf, coelomic funnel; coel, coelomic cavity; cl. o, opening of archinephric duct into cloaca; ect, ectoderm; end, endoderm; f.c, flame cell; n, nephridial tube. (Graham Kerr, 1919).

Because they are all part of the nephric or "coelomoduct" system, it does not necessarily follow that the pronephros, mesonephros and metanephros are exactly parallel structures, but there are very good reasons for believing that the three parts of the kidney system are all developments of such a primitively serial system of tubes. Again, this does not mean that their modes of development need be the same throughout the length of the body, and in fact they are obviously not identical. Each part, therefore, has to be discussed separately.

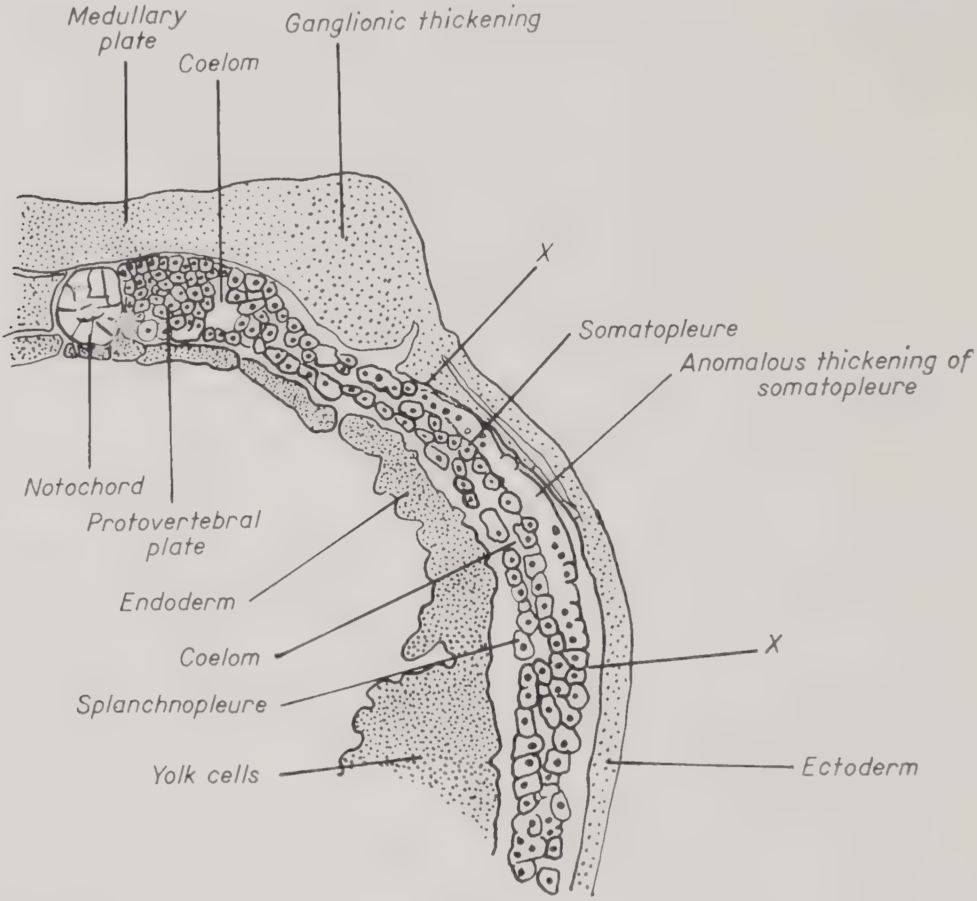

FIG. 13.19

Transverse section through the anterior trunk region of the frog about 60 hr. after fertilization. Note the anomalous thickening of the somatopleure between X and X, foreshadowing the development of the pronephros. (Redrawn from Field, 1891).

Some further points concerning the pronephros of the cyclostomes offer some very interesting food for thought. In most cyclostomes, as already mentioned, there are several open pronephric funnels leading from the pericardial cavity and joining the archinephric duct. Near the funnel openings, but quite separate from them, are the one or more large glomeruli of blood vessels projecting into the coelomic cavity. In the myxinoids the picture is somewhat more complex. Again there are "pronephric" funnels opening into the coelomic cavity, but their tubes join up to central ducts which run into and along a special blood sinus. They do not in the end pass their contents down the archinephric duct because this does not fully develop in the segments immediately behind the pronephros: instead, the ducts open directly into the blood sinus through orifices which are guarded by masses of small round and basophil cells (Conel, 1917; Price, 1910) (Fig. 13.20). There is thus a direct connexion established between the coelomic fluid of the pericardial region and the blood, by way of pronephric tubes and a portal of "lymphoid" tissue. One is reminded of the junctions made in *Phoronis* between the coelomoduct and the earlier developed nephridia, when the solenocytes of the latter change their character and float away in the blood as basophil cells. A similar development of "lymphoid" tissue occurs in the ammocoete larva of *Lampetra Planeri* (Gérard, 1933). It would be interesting to know if this foreshadows the development of lymphatic drainage channels, and, if it does so, what is the origin of the cells constituting the "cell mass" at the junction with the blood. The matter will be discussed again on p. 336. A similar system of "nephrostomes" draining back into blood channels has been described by Marshall and Smith (1930) in the elasmobranchs and it occurs also in the Anura (Nussbaum, 1886).

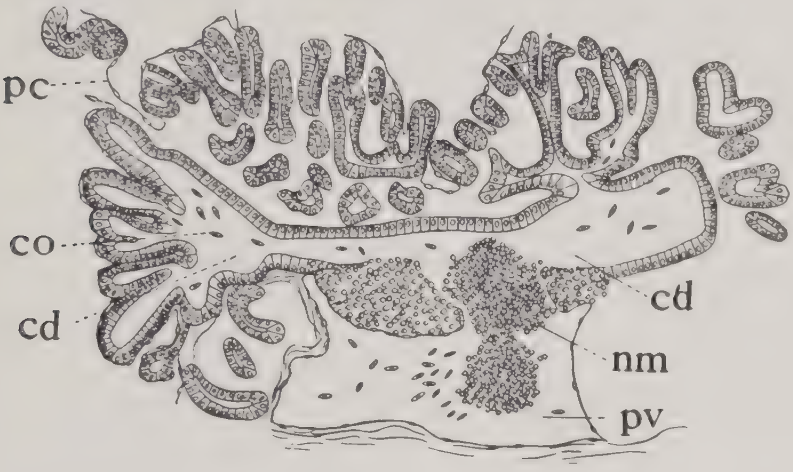

FIG. 13.20

Diagram showing the pronephric tubules joining to form a duct, which then leads through "nuclear masses" into the veins (*Bdellostoma stouti*). cd, central duct; co, blood corpuscle; nm, nuclear mass; pc, pericardium; pv, pronephric vein. (Conel, 1917).

Coelomic fluid in some animals, as in the elasmobranchs (Smith, 1929) may be regarded as being in the nature of an excretory fluid, and the development of large coelomic glomeruli may be partly connected with an excretory function. The more filtration that occurs through the glomeruli, the greater is the necessity for reabsorbing essential metabolites from the filtered fluid. The incorporation of glomeruli within the strictly renal tissue, according to some scheme like that illustrated in Fig. 13.21, is thus an obvious line of development; moreover, the opening of connexions between renal tubules and the coelom by open nephrostomes is of common occurrence in the mesonephric region of many groups, and the amphibia provide excellent examples (Fig. 13.22), but whether all glomeruli and all Malpighian capsules have the same origin, i.e. from the coelom, is a much more debatable point. Certainly, in the metanephros of the higher vertebrates, cups form on the ends of branching tubules developing in the metanephric blastema and vascular capillary knots invade these cups. In this case, the glomeruli develop directly and there is no indication that they move inwards from the coelomic cavity as such.

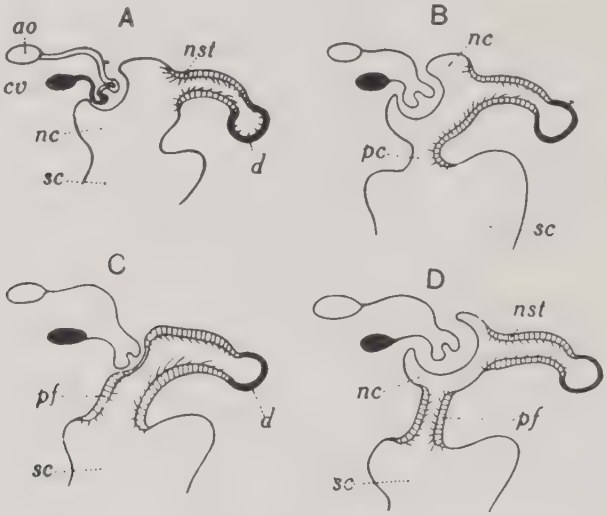

FIG. 13.21

Scheme suggested by Goodrich (1945) for the inclusion of the glomerulus within the nephron. A. Condition in pronephric tubules. B. Hypothetical intermediate condition. C. Supposed primitive condition. D. Mesonephric tubules complete. ao, dorsal aorta; cv, cardinal vein; d, archinephric duct; nc, nephrocoele; nst, nephrocoelostome; pc, peritoneal canal; pf, peritoneal funnel; sc, splanchnocoele. (Goodrich, 1945).

From all the information now available from different groups of animals, some general scheme for the development and significance of the vertebrate excretory system can be tentatively suggested. It will be realized that, in outlining this scheme, general principles are being sought from which further investigations may start. The scheme will probably never apply *in toto* to any one animal since all sorts of special adaptations in time and position of development, in function and in time of degeneration of the various parts must have occurred in order to provide the great variations which are to be found throughout the vertebrate kingdom. Nevertheless, there is clear evidence for there being an organized basic pattern, if only it could be understood.

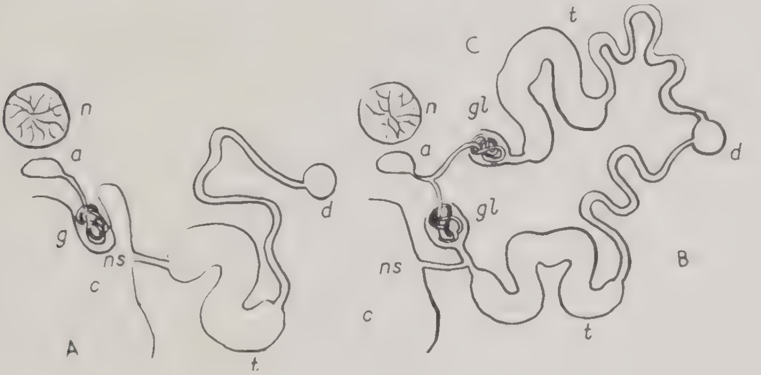

FIG. 13.22

Types of nephron actually found. A. Pronephros of cyclostomes and of amphibia. B. Mesonephros of urodeles. C. Mesonephros of selachians, teleosts, amphibia, and amniotes. a, aorta; c, coelomic cavity; d, archinephric duct; g, glomus; gl, glomerulus and capsule; n, notochord; ns, nephrostome; t, tubule. (Redrawn after Gérard and Cordier, 1933).

The diagram in Fig. 13.23, 1 shows a hypothetical primitive stage of an animal with serial coelomic cavities, serial coelomostomes and serial nephridia. In nemertines, annelids, *Priapulus* and other animals, nephridia often group together and may acquire a common duct, while in the invertebrates, coelomoducts rarely if ever fuse together. Moreover, reduplication of units by secondary branching is common in nephridia and has obvious physiological advantages. Secondary branching of coelomoducts would seem to be pointless. A second stage, therefore, could be represented by Fig. 13.23, 2, showing the fusion of the excretory pores to one posterior pore. One can imagine that the fewer points of entry or exit to and from the interior of an animal, the better from the point of view of preventing infection and of the stability of the inner environment. Figure 13.23 also shows some further possible variations on the primary theme of one coelomoduct and one nephridium per segment or unit in the series, and it will be seen that there are precedents for most of them.

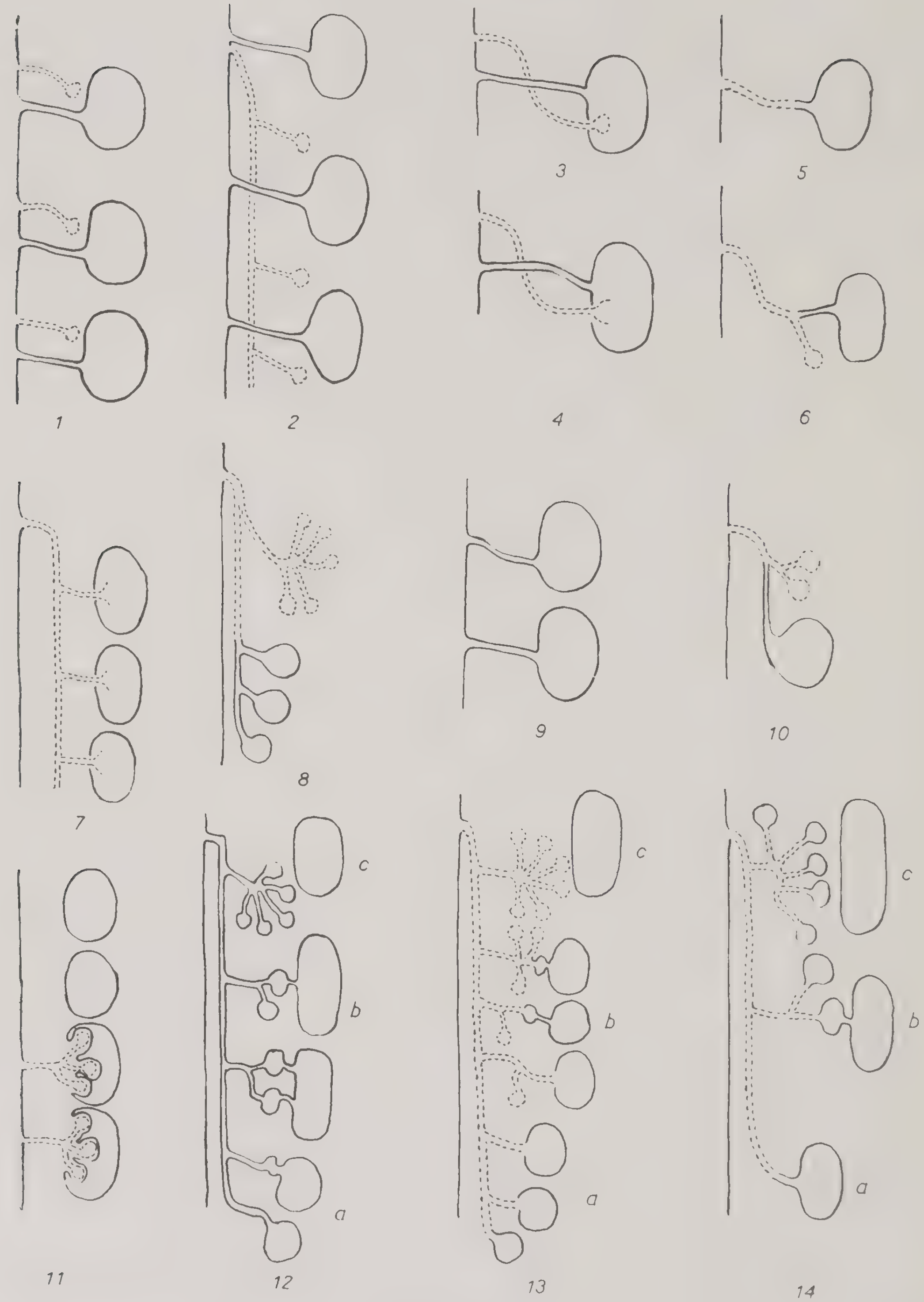

FIG. 13.23

THE NATURE OF THE CELLS IN THE EXCRETORY TUBES OF THE VERTEBRATES

In these nephridial tubes and coelomoducts there are found to be several forms of cell. In the coelomostome the cells are often ciliated or have long flagella, and the cells at the other end of the coelomoduct tube tend to be similar to, if not identical with, those of the general surface of the animal. It has been suggested that the cells nearest the coelomic cavity have powers of regulating the composition of the coelomic fluid as such, but how far the cells of the coelomostome differ from those of the general coelomic surface in this way is not clear. In so far as the coelomic fluid becomes in some animals an excretory fluid, it is probable that secretory or absorptive activities must be assigned to some cells in the system. It may be remembered that in amphibia the whole coelomic lining of the female may be composed of ciliated cells, and even in man it is not an uncommon pathological event for ciliated pockets to develop in the pelvic region of the coelom. Nephridia may have solenocytes, or like coelomostomes they may have cells with long flagella if they open by nephrostomes into the coelom. In addition, there are often granular "secretory" cells lining the nephridial tube, though the function of these may equally well be reabsorption and in many cases undoubtedly is so. Athrocytosis, or the collection of dye particles, lipoid globules, proteins or other micellar or particulate matter by the cells, is also a phenomenon which is sometimes associated with the coelomic epithelium (e.g. in Chelonia, Delaney, 1929) and with coelomic funnels, and these latter often act as

FIG. 13.23

Some possible and actual arrangements of nephridia ( ) and coelomoducts (——). 1. Primitive serial arrangement of coelomoducts and nephridia e.g. nemertine. 2. Serial coelomoducts. Nephridia with common duct, e.g. nemertine. 3. Serial nephridia (closed) in connexion with serial coelomic cavities; separate coelomoducts e.g. annelids. 4. Serial nephridia (open) in connexion with serial coelomic cavities; separate coelomoducts e.g. annelids. 5. Mixonephridium e.g. annelid (*Arenicola*). 6. Protonephromixium e.g. annelid (*Phyllodoce*). 7. Open nephrostomes with common nephridial duct e.g. annelid (*Hoplochaetella*). 8. Coelomoduct fusing with nephridial system e.g. *Priapulus*. 9. Disappearance of nephridial system (? *Balanoglossus*). 10. Coelomoduct grafted on to nephridial canal e.g. (*Phoronis*). 11. Nephridia in connexion with coelomic cavities; other coelomic cavities closed e.g. *Amphioxus*. 12. Supposed condition in vertebrates (a) Pronephros (b) Mesonephros (c) Metanephros. Subdivision of coelomic cavities with disappearance of nephridia. 13. Another possible interpretation of the system in vertebrates as combination of nephridial and coelomoduct systems. Bowman's capsules as ends of nephridial tubes. 14. Another possible system for vertebrates, with Bowman's capsules and perhaps part of nephrons as of coelomic origin.

collecting grounds for the various coelomic phagocytes. This cannot be used to distinguish between coelomostome and nephridiostome, however, since phagocytic cells are associated with the nephridiostomes of many oligochaetes, and the middle segment of the nephridium of *Lumbricus* possesses strongly developed athrocytic powers (Cordier, 1933).

In considering the nature of the cells that constitute the mammalian kidney, and, in trying to evaluate the manner in which the organ develops and functions, it is necessary to approach the problem without prejudice and from several different directions, but, as suggested earlier, the nature and the orientation of the constituent cells are probably of more significance than the pattern of embryological development.

From a study of phylogeny, the kidney tubes of vertebrates could be the direct descendants of coelomoducts, or of nephridial systems or some combination of both. As we have seen, remnants or specializations of both systems are found among the animals that are thought to be related to primitive vertebrates. The archinephric duct on these grounds is perhaps more likely to be an inheritance from nephridial systems, and the repeated branching of the tubules of the metanephros is also a nephridial character. On phylogenetic grounds, the "nephrostomes" could be interpreted either as coelomostomes or as open nephridiostomes, since there are precedents for nephridia opening into the coelom.

On embryological grounds, there has been a tendency for some years to regard the kidney as a modified coelomoduct system, and as having nothing to do with nephridia. This view is largely based on the development of the pronephros as it appears in the amphibia, where the pronephric tubules grow out from what are apparently, though by no means certainly, mesodermal pouches and then join up with the archinephric duct, which itself has been seen to develop from similar more posteriorly placed pouches and to grow backwards towards the external opening. The force of this argument is largely lost, however, when in elasmobranchs and mammals the duct can be seen to arise from cells which leave the ectoderm and then, within the mesenchyme, to group together and to grow backwards to form the archinephric unit, which is then joined by the outgrowths from the coelomic pouches. Moreover, the argument that "because it elongates in a caudal direction to open eventually on to the outside and because it does not develop forwards from an ectodermal invagination the archinephric duct is not nephridial," has no weight for two reasons. First, in those annelids which have a common nephridial duct this must develop caudally as the animal elongates and adds further segments to its length. Second, although protonephridia undoubtedly started as true invaginations of the ectoderm, this method was quickly superseded by the segregation of certain epidermal cells at an early stage of development as nephridial cells which then immediately penetrated the mesenchyme and only later divided and produced nephridial tubes, flame cells and nephridiostomes. The development of the archinephric duct is thus quite consistent with its having a nephridial origin, and the manner in which its cells originate in cyclostomes, elasmobranchs and amphibia would be worth further investigation with this in mind. On the other hand, the outgrowths from the coelomic cavities and the open ciliated funnels which they produce in the pronephros and sometimes in the mesonephros are strongly reminiscent of the coelomic funnels (coelomostomes) at the mouths of coelomoducts, though to prove on developmental grounds that they are not nephridiostomes would not be easy. Embryologically the evidence is conflicting. Sometimes tubes or cords of cells push outwards from the tubes leading away from Malpighian bodies and grow back towards the coelom and then open into it (Gray, 1933). On the other hand, there is also evidence for the development of similar tubes in exactly the opposite direction, i.e. starting from the coelom and joining the neck tubes of the Malpighian capsules (Fraser, 1918). As hinted above, the direction of growth of a structure may be helpful in providing a clue to its origin but a change in the relative time scale of development of two parts may often obscure the original pattern as it has done with the nephridial tubes in annelids. One has only to consider the variety of ways in which the mesenchyme may arise in the different phyla to see how little the actual course of development is rigidly determined. From this point of view, the nature of the blastemata which form the mesonephros and metanephros is of some interest. They certainly appear to have an origin separate from the archinephric duct to which they later attach themselves, but the nephridial rudiments in worms, which attach themselves to a common nephridial canal, also have an origin different from that of the canal, so this separate origin would offer no clue as to whether either or both correspond to nephridial or coelomostome structures.

From the point of view of cell physiology there are several reasons which perhaps suggest that the proximal tubule of the kidney should be associated with coelomic structures rather than with nephridial. First, if it is legitimate to generalize from the figures secured by analyses of the fluid obtained by the micropuncture techniques from the tubules of the amphibian kidney (Walker, Hudson, Findley and Richards, 1936; Walker and Hudson, 1936a, b, c; Montgomery and

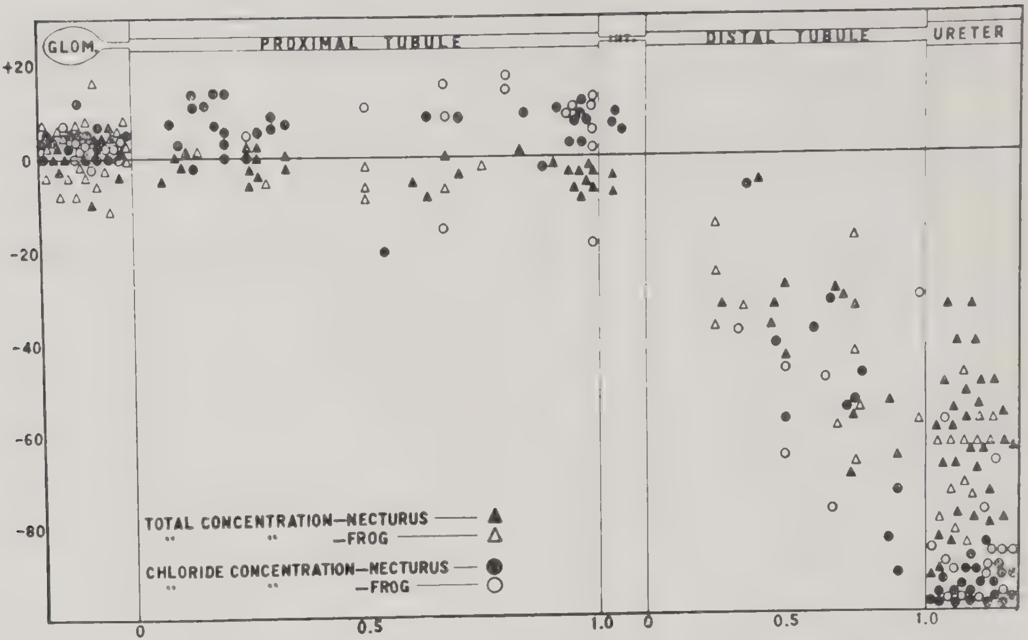

FIG. 13.24 (*a*)

Chart showing differences between blood plasma and fluid collected from various levels of the renal tubules with respect to total concentration and chloride concentration. Abscissae: Distances along the proximal and distal tubules as fractions of the total length of each. Ordinates: Zero = plasma concentration level. Points for chlorides above and below zero represent percentage increases or decreases. Points for total concentrations give a measure of the difference in osmotic pressure between plasma and tubule fluid. (Smith, 1951).

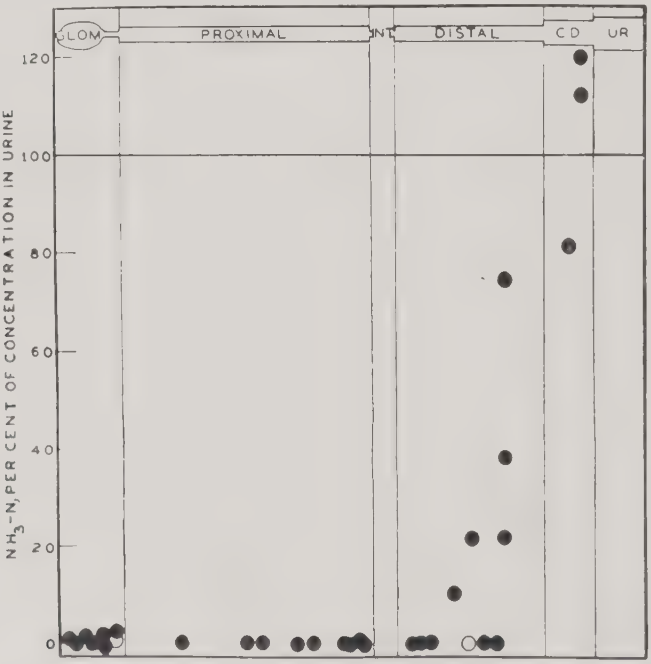

FIG. 13.24 (*b*)

Similar observations on the ammonia concentration in the urine in the different tubules, as compared with the ammonia in the urine. (Walker, Hudson, Findley and Richards, 1936).

Pierce, 1936), there is the rather remarkable constancy of the osmotic pressure, chloride concentration (Fig. 13.24 a, b) and the hydrogen ion concentration of the tubular contents in spite of the complete removal of glucose from the fluid and of various other subtractions and additions e.g. urea, phosphate and probably water. This "homoiostasis" of the tubular fluid is more in harmony with the fluid being essentially coelomic fluid than with its being the excretory fluid of a nephridial tube. It has, however, been pointed out (Ramsay, 1949) that the osmotic pressure of the fluid in the first segment of the nephridium of *Lumbricus* is also similar to that of the surrounding fluid so that it might seem at first sight that the proximal tube was more like the beginning of a nephridium. In the nephridium, however, the proximal segment seems to be fairly freely permeable: it is very thin-walled, and the osmotic equilibrium is probably passive. In the proximal tubules of vertebrates, the histological appearance suggests, and experiment confirms, that active absorptive processes are occurring, and osmotic equilibrium must therefore be at least in part an "active" phenomenon. This constancy of the internal fluid suggests that the cell-activity is dominated by the contents of the tubule rather than by the composition of the body-tissues. Quite the opposite happens in the rest of the tubule and the regulation, in that part may lead to wide fluctuations in urine composition, presumably in an attempt to maintain the constancy of the tissue fluids. In this connexion it may be significant that the two main hormones affecting kidney function, namely, aldosterone, or whatever turns out to be the physiologically circulating mineralocorticoid from the adrenal cortex, and the antidiuretic hormone of the anterior pituitary, are thought to exert their effects on some part of the kidney other than the proximal tubules, e.g. the distal tubule, the loop of Henle or the collecting tubules. Their concentration in the blood is presumably the final determining factor. The fact that the water-controlling principle of the pituitary also affects the permeability of the skin in amphibia might perhaps be taken to indicate an epidermal origin for those parts of the kidney which it affects. On the other hand, the action of vasopressin and oxytocin on other contractile tissues of the body, may indicate that the water-controlling factor affects the shapes of cells and the sizes of spaces between the cells as in blood vessel walls, or in the thin segment of the loop of Henle (Wirz, 1957) in the kidney rather than alters the rate at which cells "secrete". That water elimination or salt conservation are functions to be ascribed to the distal part of the nephron is suggested by the observation (Grafflin, 1937a) that marine teleosts, some of which may have aglomerular kidneys, tend not to have distal tubules whereas these are generally found in fresh-water teleosts. The teleosts are, in many respects, an aberrant group, so this observation may have little bearing on the general problem, but it does nevertheless raise awkward questions about the homology of the proximal tubule with the coelomoduct and about the mechanism by which homoiostasis is maintained in the marine fish if the luminal surface of the proximal tubule cell is the dominant surface of the cell. The fact that all the urine is 'secreted' is, of course, consistent with derivation from the coelomic epithelium. In the earthworm the thick-walled distal segment of the nephridium is actively concerned with absorbing salt and large osmotic pressure changes occur in the fluid in this segment (Ramsay, 1949).

Second, the cells of the proximal tubules have athrocytic powers which are apparently not shared by other regions of the kidney, and from this point of view it may be remarked that there is generally a gradient of these powers along the tubule—small particles being picked up by cells nearest the glomerulus, and larger particles further along the tube. Trypan blue may appear as blue particles near the glomerulus and red particles at the distal end of the proximal tubule (Cordier, 1928). This is, however, not a strong argument since ability to display athrocytic powers must depend on the presence of suitable particles to be picked up, and these may never be presented to the cells of the distal tubules since the proximal tubules work so efficiently; though it may be noted that distal tubule cells do not pick up protein from the lumen when this has passed the glomeruli in renal disease. Moreover, the tubule cells and paranephrocytes in the protonephridia of turbellarians are capable of athrocytosis (Reisinger, 1922-3) and these cells are at the very foundation of nephridia. Athrocytosis occurs also in the middle segment of the nephridium of *Lumbricus* (Cordier, 1933; Cuénot, 1898). The direction from which the particles are picked up by the cells is, of course, important and not always clear.

The gradient in the proximal tubule is again shown in the distribution of alkaline phosphatase, which becomes less active towards the distal end of the tube. Alkaline phosphatase is not usually present in the distal tubules. Once again, however, the presence or absence of this enzyme is not generally a very useful guide as to the nature of the tissue. Its distribution is somewhat capricious. Nevertheless, it is a point of some interest that the nephridial tubes of both *Geonemertes* and of the triclad *Rhynchodemus* (Danielli and Pantin, 1950) both show evidence for the presence of alkaline phosphatase in the ciliated and convoluted parts of the tubules though not in the solenocytes or in the more glandular part of the excretory tube which is present in *Geonemertes*. In the kidneys of vertebrates the presence of this enzyme has been correlated with glucose absorption, but there is really no good evidence that this is its true function. Data on its distribution in the nephridia of worms from other types of environment might throw light on its function. Since aglomerular teleosts possess the enzyme in their kidney tubules, glucose reabsorption does not seem to be its most probable function. Moreover, its connexion with salt balance is perhaps indicated by the fact that the amount in the kidney cortex decreases after adrenalectomy and increases in response to deoxycorticosterone (Folley and Greenbaum, 1946).

The fact that glucose 6-phosphatase, on the other hand, occurs only in the proximal tubule (of mammalian kidneys) (Chiquoine, 1953; Wachstein, 1955) may indicate a coelomic origin for this tissue, though it could, of course, be regarded merely as an adaptation by the tubule cells to the use of a filtration method for producing the basic urinary fluid which then has to have its glucose removed. In the kidney of the rat a yellow, iron-containing pigment sometimes appears in the cells of the proximal tubules (Grafflin, 1942), and its distribution suggests that the cells in the final third of the tube are somewhat different from those of the rest of the tube. The injection of ferric salts into the circulation in elasmobranchs (Edwards, 1933a) also results in their being picked up in the terminal third of the proximal tubule. In certain fishes also (Edwards, 1933b), the final part of the proximal tubule contains cells which appear hyaline and undergo changes in appearance in connexion with the "nest-building", and therefore presumably with the sexual activity of the animal.

If it is possible to draw a broad picture of the proximal tubule it would suggest that the tube has more or less of a gradient system throughout its length, starting with a neck segment whose cells may be strongly ciliated or flagellated as in *Muraena helena* (Edwards, 1933b) and ending with phagocytic or even mucin-containing cells in its final portion as in *Danio*. Thus it contains within itself a pattern of cells similar to that of the original "blastula-like" organism, and also similar to that of the primitive nephridium. In other words, its cells are organized in sequence for purposes of ionic balance. Sometimes the gradients are sharp and steep with rapid transitions from one cell type to the other. In other animals there is a progressive and gradual change throughout the length of the tube; in still others, like the common eel (Grafflin, 1937b), brush-border cells intermingle with cells whose internal surface has bulbous projections, and ciliated cells occur scattered throughout the tube. In this animal, therefore, the regular gradient appears to have been lost but the cells from the different parts of it are mingled together. A similar situation occurs in other systems where ionic balance is a primary requirement, e.g. in the choroid plexus, Fallopian tube, and to some extent in salivary glands. The existence of a gradient in itself or of the several types of cell, manifestly tells nothing decisively of the origin of the tubes, but it allows parallels to be drawn with other similar systems and further investigation into the characteristics of the cells in the different regions, e.g. their affinities or reactions to such things as steroids and other hormones, etc., may then establish their relationships more firmly. This theme will be pursued in a later chapter (see p. 316).

From the point of view of the micro-structure of the cells, little positive contribution can be made. The brush border, the interdigitations of the cell membranes of the proximal tubules (Foote and Grafflin, 1942) and the complicated infolding structures of the basal end of the cells of the distal tubule in particular are certainly characteristic (Pease, 1955), but a brush border is seen also in intestinal cells and probably both it and the infolded basal surface reflect directional movements of water or dissolved substances across the cells more directly than they do the origin of the cell (Pease, 1956). On the other hand, a brush-border-like structure is also associated with the flagellate cells which constitute the bulk of the excretory cells forming the tubules of the aglomerular kidneys of certain marine fish (Defrise, 1932). In this case, reabsorption is unlikely to be a major property since all the constituents of the urine are thought to be secreted.

It is a point of some interest that the cells in the Malpighian tubules of certain insects (e.g. Orthoptera) have a fine structure very like that of the proximal tubules in mammals (Beams, Tahmisian and Devine, 1955); this presumably means that the cells selected for this function are biologically similar to those selected for excretion in other animals and that they have undergone similar modifications because they are adapted to similar functions, unless the Malpighian tubes are, in fact, some direct derivative of an earlier coelomoduct-nephridial system.

It may be concluded then that the kidney tissue of the higher vertebrates is unlikely to be entirely of coelomoduct tissue as was at one time thought, and that some sort of nephromixium is really a better description of the nephron. The differences between coelomostome cells and nephridial cells caused by their different embryological origins may, in fact, be very much smaller than was at one time supposed. Both groups of cells are phylogenetically very old. Both are, and have always been, set aside for the regulation of the composition of tissue fluids, and both systems probably contain cells which, like the cells of the early blastulae, are graded, more or less sharply, into two camps according to their ionic relationships with their surroundings. Both systems show propensities for tubule formation, but how these tubes form and connect with each other in the higher vertebrates may be very different from the primitive arrangement. In the pronephros of *Petromyzon* (Nussbaum, 1886) and of the gymnophionan (*Hypogeophis*) (Brauer, 1902), the pronephric tubule is relatively simple and consists of a ciliated segment followed by a "brush-border" segment. The mesonephric tubule may also have this simple arrangement or it may, on the other hand, have the system more or less duplicated. In some reptiles, a ciliated segment leads into a brush-border segment, this leads again to a ciliated segment opening into another "glandular" segment often divided into two parts, the more distal of which sometimes, as in snakes, varies in structure with the sex of the animal, and thence to the duct (Regaud and Policard, 1903 a and b; Cordier, 1928; Herlant, 1933). Has this developed *ad hoc* by cells specially differentiating for particular tasks? or is it really repetitive, i.e. two coelomoducts joined end to end? or does it signify coelomoduct opening to nephrostome in some way? It would be informative to ascertain how the activity of each type of cell is controlled. As this pattern of kidney is followed, with variations, in all the higher forms, adequate answers to these questions might solve many problems. For the present, however, a consideration of individual kidney cells in relation to their position in the scheme of cells shown on p. 219, and in relation to the dominant pole of the cell may help to clarify certain problems of renal physiology. In the kidneys of mammals ciliated cells are not normally found. Presumably the "gradient" required in their kidney tubules no longer covers such a wide range of concentrations since, in mammals, homoiostasis of the blood composition has been greatly improved. Nevertheless, in tumours caused in the kidneys of hamsters by the implantation of stilboestrol, perfectly good ciliated cells are abundant (Mannweiler and Bernhard, 1957) (Fig. 1.1.)

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CHAPTER 14

*COELOMODUCTS, NEPHRIDIA AND GONADS

IN the last chapter the coelomoduct and nephridial systems were discussed as the possible fore-runners of kidney tissue, and the capacity of both the coelomic epithelium, including its ducts, and of the nephridial tubes to modify the composition of the coelomic fluid and of the blood or tissue fluid respectively was emphasized. The original function of the coelom and its ducts was not, however, given more than a passing mention. Its primary function was, of course, to house, nurture and eventually to liberate the special totipotent cells of the organism, the germ cells. The origin of these germ cells is embryologically not always very certain and it is possible that coelomic epithelial cells as a whole may, at least in some animals, retain their primitive characteristics as originators of the germ cells. It will be remembered that the tokocytes of the sponges are derived from the amoeboid cells of the posterior half of the embryo, and that the amoebocytes of the sponge also have the same origin. In many animals the germ cells undoubtedly arise in the endoderm (Schapitz, 1912) and are early segregated as special cells. In the crustacean, *Tisbe furcata* (Fig. 14.1), it is interesting to find that they arise from two cells occupying the same relative position in this embryo (Witschi, 1951) as the archaeocytes in the sponge embryo, *Clathrina blanca*. However early they become established as a separate group of cells, the germ cells in higher animals are sooner or later shed

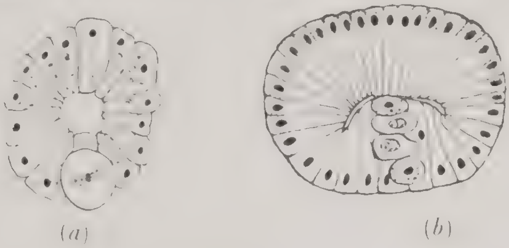
(*a*) (*b*)

FIG. 14.1
Origin of germ cells in the crustacean *Tisbe furcata*. (*a*) 63-cell stage with "archaeocyte" at the posterior pole (cf. Fig 8.3). (*b*) Gastrula stage, when the archaeocyte has divided to give rise to two endoderm cells (with yolk granules) and two germ cells (open nuclei). (Redrawn, after Witschi, 1951).

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* "Coelomoduct" = generic term for tube connecting coelom to outside. Coelomoduct = *outgrowth* from coelom to outside, as opposed to nephridium.

into the coelom or into ducts in connexion therewith, and eventually passed to the outside world through the coelomoduct or related channel. In the discussion on the kidney, it has been shown that in this organ different parts of the modified coelomoduct-nephridium complex have different functions with regard to the water and salt content of the fluid in the lumen, and that in the proximal tube, for example, which probably corresponds with the true coelomoduct, there is a gradient of activity along its length, and the actual coelomostome region may differ structurally from the tubular region, as it does, for example, in the pronephric tubules (see Brauer, 1902). Thus, if the germ cells became prematurely housed in these tubes instead of in the main coelomic cavity, they would necessarily become subjected to a special intratubular environment and might well, by analogy with the behaviour of the amoeba (*Naegleria*) in response to changed environmental conditions, have their subsequent differentiation materially affected according to the composition of the fluid in the particular part of the tube in which they became lodged. Apart from this, however, the early passage into the tubes might well be advantageous on other grounds. As the coelomic cavity increased in importance as the main body-cavity, the composition of its fluid might be subject to greater variation, particularly since the coelomic fluid came to be used as an intermediary between blood and excretory organs by the development of glomeruli on the coelomic wall; secondly, a more exact composition could be maintained by specializing the cells of the duct itself.

The mature sex glands of mammals do not, at first sight, seem to offer much support for the sort of hypothesis outlined above and appear as very specialized structures with their own peculiar systems and arrangements of cells. An examination of their embryological and phylogenetic development (see Fig. 14.2) however, and a rather more searching enquiry into the intimate cellular behaviour put a rather different complexion on the matter, and a study of the comparative aspects of their physiology reinforces the new look.

In general, the gonads of vertebrates develop from the coelomic wall just medial to the developing mesonephric material and, in the male, a direct continuity is secondarily established and persists between the testis tubules and the mesonephric tubes. The gonad material tends to occur in segments posterior to the pronephros and to become more and more concentrated in the middle segments of the body in the region of the mesonephros. In relation to the argument which will be developed, it is pertinent to notice that the segments immediately behind the pronephros seldom develop true renal units. For example, in the dipnoan fish *Protopterus*, there are no renal units between the

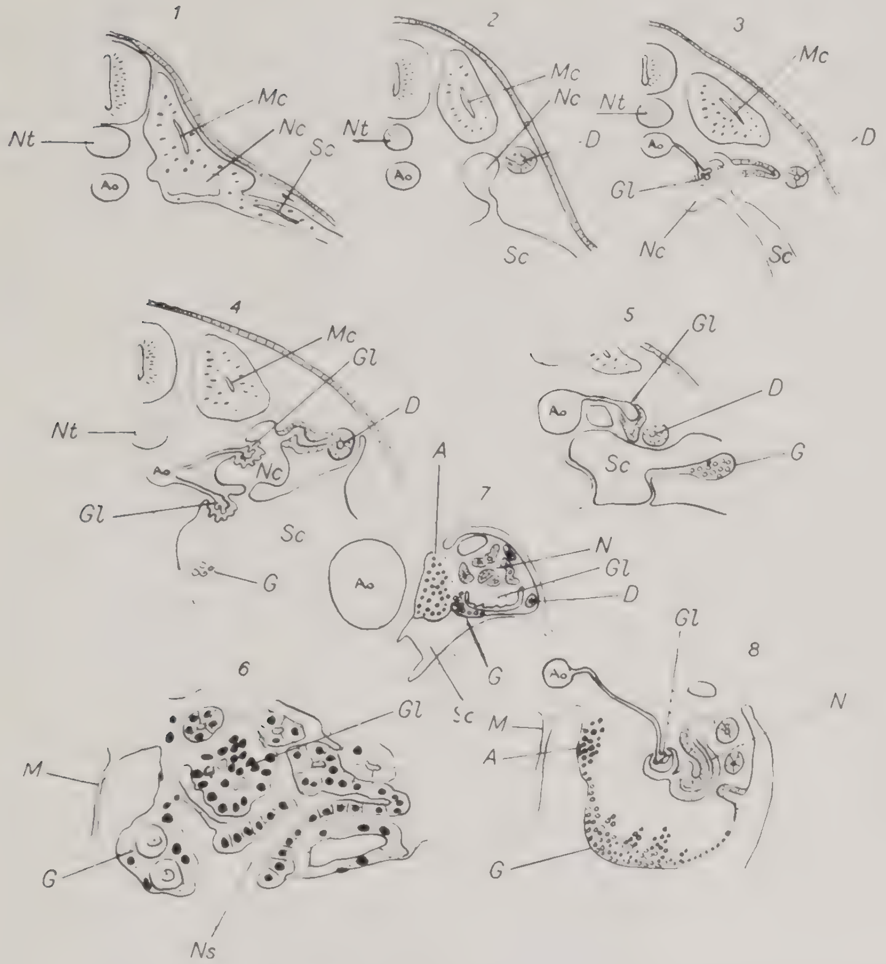

FIG. 14.2

Diagrams to illustrate the relationship between the nephric, gonadal, and adrenal tissues in the vertebrates. 1. Diagram illustrating the subdivision of the coelom into myocoele, nephrocoele, and splanchnocoele. 2. Diagram illustrating the development of the archinephric duct in relation to the nephrocoele. 3. Diagram illustrating the formation of a glomerulus in the nephrocoele and renal tubules approaching the archinephric duct. 4. Diagram illustrating glomeruli developing both in the nephrocoele (internal) and in the splanchnocoele (external), and the position of the gonadal rudiment. 5. Diagram illustrating the position of the gonadal rudiment in the cyclostome with reference to the nephric structures. 6. Diagram illustrating the position of the gonadal rudiment in the frog with reference to the nephric structures. Note the open nephrostome and the channel leading into the capsule of the glomerulus. 7. Diagram illustrating the relative positions of nephric, gonadal and adrenal rudiments in the marsupial. 8. Diagram illustrating the relative positions of nephric, gonadal and adrenal rudiments in the mammals. A, Adrenal; Ao, Aorta; C, Coelomic cavity; D, Archinephric duct; G, Gonad; Gl, Glomerulus; M, Mesentery; Mc, Myocoele; N, Nephric tissue; Nc, Nephrocoele; Ns, Nephrostome; Nt, Notochord; Sc, Splanchnocoele.

segments 5 and 20. In most vertebrates, the first signs of the developing gonad are paired thickenings of the dorsal splanchnocoelic epithelium and a tendency for the cells from these thickenings to penetrate the underlying mesenchyme in cord-like masses (Fig. 14.3). The gonad rudiments are derived from cells of the coelomic epithelium and the mesenchyme in the dorsal region of the splanchnocoele (Fig. 14.2): they are thus occupying a position almost identical with that of the nephric rudiments though rather nearer to the mid-line. In this region of the coelomic wall the epithelium does not rest on a basement membrane (Torrey, 1945); coelomic cells remain in direct contact with underlying mesenchyme and continue to penetrate it. Large round cells—presumably the potential germ cells—may be conspicuous among these masses of other cells. The cord-like invading masses gradually lengthen and become defined as the primary sex cords (Fig. 14.4). As development progresses, the mass of cells in the gonad becomes broadly divisible into two regions, a cortical part and a medulla. In general, at this stage, the gonad is "indifferent", neither demonstrably male nor female, and may continue so for a considerable

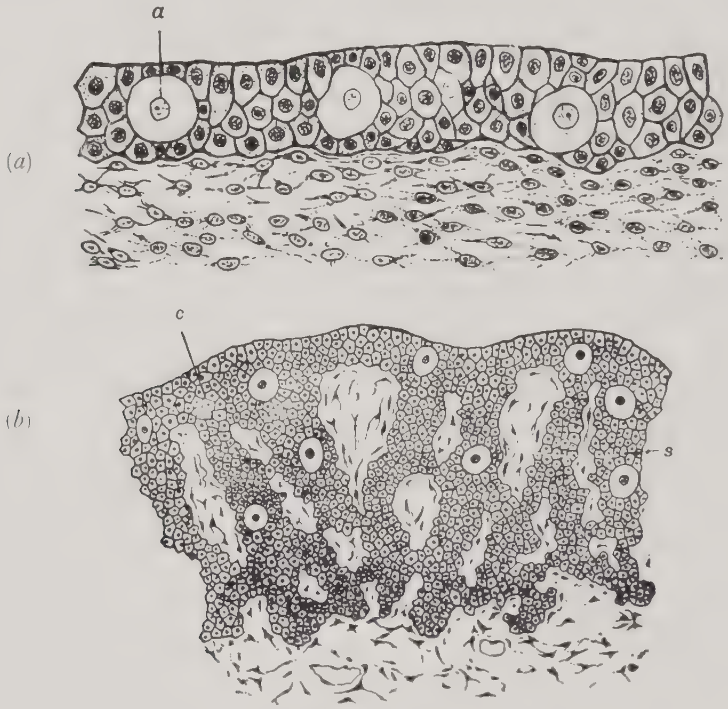

FIG. 14.3

Primordium of the gonad in the early indifferent stage. (*a*) Thickening of the germinal epithelium. (*b*) Formation of sex cords. a, primitive sex cell; c, cortex; s, sex cords. (Maximow and Bloom, 1952).

time. When, however, the sex becomes established, the medulla is the male region and the cortex the female. In many vertebrates, ovo-testes are of normal occurrence during the development of the animal, and

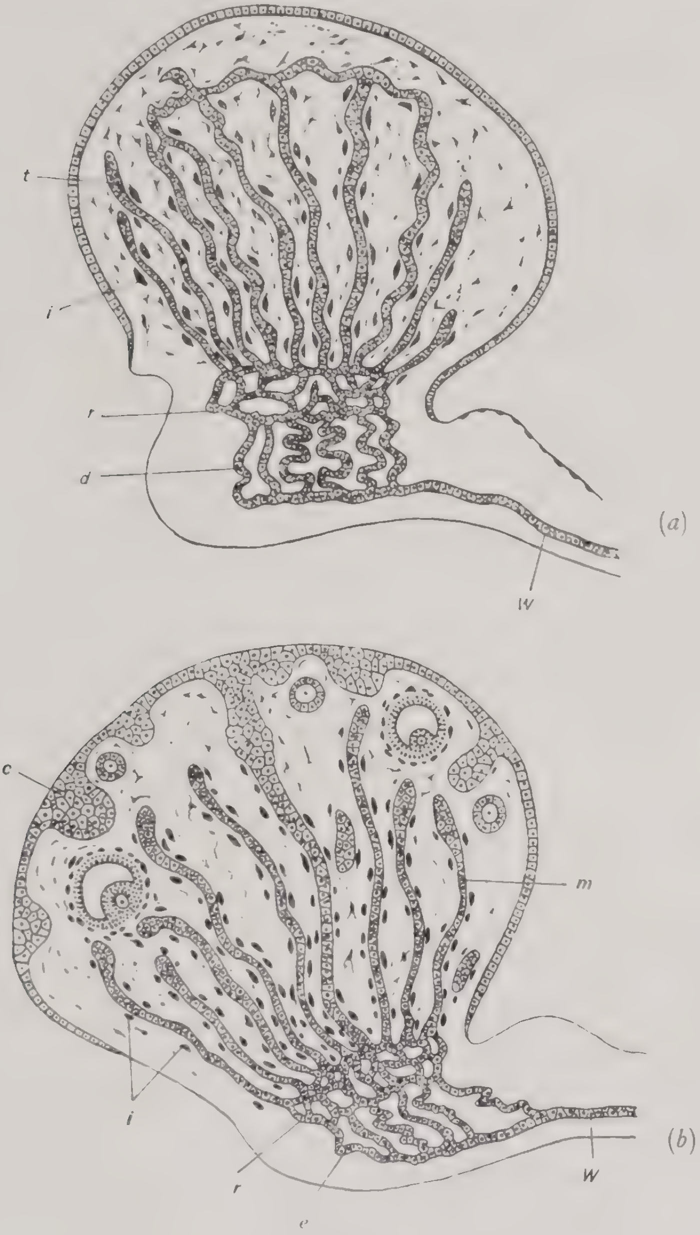

Fig. 14.4

Diagram of developing testis (*a*) and ovary (*b*). c, cortex; d, ductuli efferentes (urogenital junction); e, epoophoron (urogenital junction); i, interstitial cells; m, medullary cord; r, rete testis and rete ovarii; t, seminiferous tubule; W, Wolffian duct. (Maximow and Bloom, 1952).

may even be functional as such. They show this same relationship (Figs. 14.5 and 6).

In the male, the cords hollow out as the definitive spermatic tubules. In the female, on the other hand, the primary sex-cords only occasionally, as sometimes happens in the opossum (Morgan, 1943) become tubular; they normally tend to disintegrate as such, though

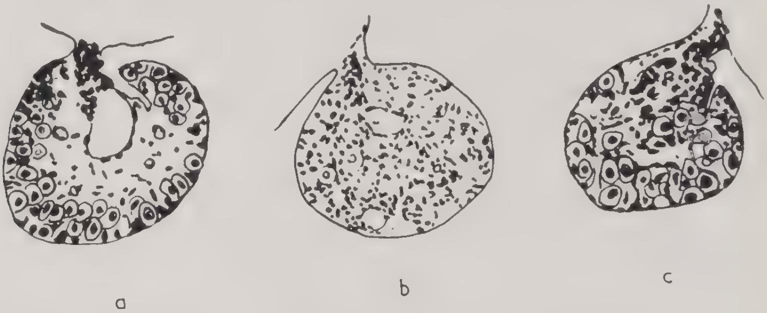

FIG. 14.5
(a) Ovary of *Rana sylvatica* (33 days old) Note development of the cortex. (b) Testis of *Rana sylvatica* (33 days old) Note development throughout the medulla. (c) Hermaphrodite gonad of *Rana sylvatica* (33 days old) with germ cells in both cortex and medulla. (Redrawn after Witschi, 1929).

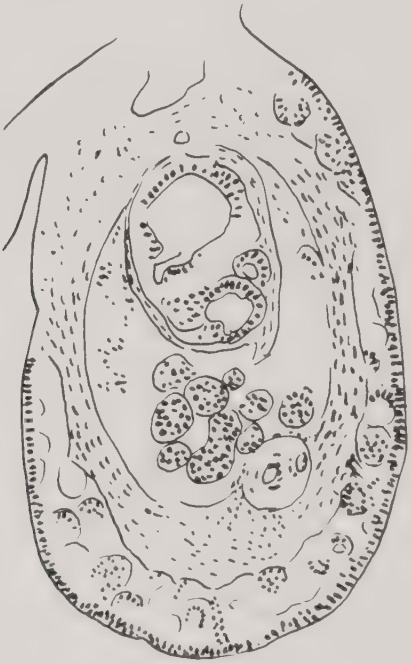

FIG. 14.6
Ovotestis of a male opossum (30 days old) after treatment with female hormone. Medullary zone is separated from the sterile cortical zone by a thick fibrous tunica albuginea. Note germinal epithelium as in the ovary. (Diagrammatic, from Burns, 1955).

the constituent cells may remain in the ovary as interstitial cells. Further ingrowths of cells from the coelomic wall occur, however, and these form the secondary or cortical sex cords. These then develop rather differently, as follicles, enclosing the individual maturing germ cells (Mossman, 1937, 1938). After a time, the follicles under the influence of gonadotrophins (Hisaw, 1947) accummulate fluid to such an extent that the germ cells can eventually escape, secondarily and by rupture of the follicle wall, back into the main coelomic cavity, to be conveyed later to the outside by the Mullerian tube which is probably a derivative of the original pronephric duct. In the male, on the other hand, the sex cords normally make connexion with the mesonephric structures and eventually the spermatozoa, in most species, reach the exterior by passing through the rete testis, vasa efferentia, and the mesonephric duct, which are all parts of the original "coelomoduct" system. Corresponding connexions between the primary sex cords and mesonephric structures occur temporarily also in the females of some species, but the so-called rete ovarii is an unstable structure and normally disappears.

The origin and homologies of these various structures constitute both an intriguing and an intricate problem, the correct solution of which would undoubtedly lead to a much better understanding of many of the peculiarities of the reproductive system.

A tendency towards the formation of tubular outgrowths by the cells in the wall of the coelom is probably as ingrained a property of these cells as any (Gruenwald, 1942), and is an heirloom from the most primitive coelomic cavities which housed the germ cells and then allowed them to escape to the exterior by outgrowing coelomoducts. It may show itself at any time, as, for example, when the removal of the pronephros of *Amblystoma* leads, among other effects, to the formation of new nephrostomal funnels from the coelomic epithelium (Howland, 1921). In the marsupial (*Trichosurus*) grooves in and invaginations of the epithelium of the genital ridge are of frequent occurrence (Fraser, 1918) (Fig. 14.7). Even in man, it is not uncommon to find out-pockets lined with ciliated epithelium developing from the coelomic epithelium in the pelvic region. As discussed in the last chapter the cells which line the "coelomoducts" must be endowed with powers of regulating the osmotic pressure, the composition and the ionic content of the fluid in the tubes, if the composition of the coelomic fluid is not simply to be that of the external fluid or of the blood filtrate. Moreover, if this regulating power is there at all, it is more than likely to work on a two-way system, i.e. concentration by some parts or by some cells, dilution by others. Gradients of activity have been shown to be a feature of the proximal tubule of the kidney and similar gradients probably occur among the cells of the sex cords also. Though originally the tubular outgrowths may have been segmental in arrangement, there is nothing sacrosanct in that arrangement. Both the branching tubules of the mammalian metanephros and the multiplication of nephridial units in the oligochaete *Megascolex* (see Fig. 13.14) can be taken as precedents for systems, which were originally segmental, and which have been converted into multiple repetitive systems.

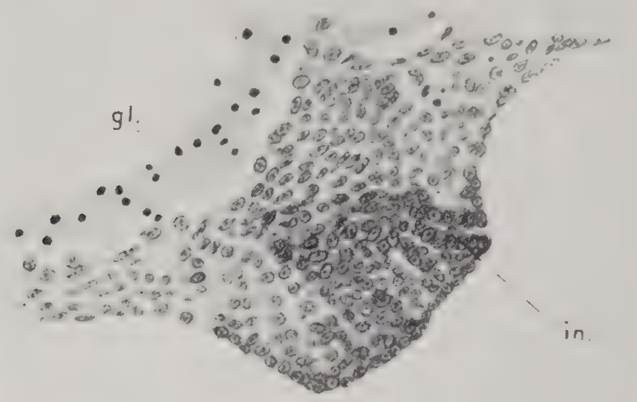

FIG. 14.7

Transverse section of the genital ridge of *Trichosurus vulpecula* showing a hollow invagination (*in*) from the coelomic epithelium into the rete strands. gl, glomerulus. (Fraser, 1918).

It thus seems to be a not unreasonable proposition that the sex cords are really outgrowths of coelomic epithelium or potential coelomoducts in a modified form, and there is some suggestion that the primary, or medullary, cords and the secondary or cortical cords may be originally, or may easily become, somewhat different in character. In terms of the original coelomoduct, the primary cords might correspond to the tubular part of the coelomoduct, and the secondary cords to the coelomostome itself. The cells of the latter, it should be noted, are frequently flagellated or ciliated and thus directly comparable with the cells of pronephric funnels generally and also with the neck cells in the kidneys of reptiles, for example. It is a well known morphological observation that "cords" of cells are often found where tubes have been present in more primitive animals, and in embryology they often precede the formation of a tube, as, for example, in the case of the archinephric duct. Whether the tube becomes patent or not may depend on a variety of circumstances.

The idea of considering the sex cords as modified coelomoducts, while attractive in many ways, probably does not cover the whole problem. There is evidence from embryology and phylogeny that the original coelomic cavity has undergone many variations in form and function. For example, in the embryos of vertebrates there is fairly clear evidence for a subdivision of the original cavity of each segment into three more or less separate regions, a myocoele, a nephrocoele and a splanchnocoele which may, however, remain connected by narrow canals, which are sometimes ciliated (Fraser, 1950); there is further evidence that glomeruli may become enclosed in pockets of the coelom, and that these pockets (nephrocoeles) may remain connected with the main splanchnocoele by ciliated ducts (Fig. 14.2). Such ciliated ducts are obviously different in origin from true coelomic funnels that develop at the mouths of coelomoducts, but physiologically their cells may share many common features since they are both immediate derivatives of the same part of the coelomic epithelium. The occurrence of cilia is presumably one such feature, and it may be mentioned again that in female frogs the whole coelomic epithelium may be ciliated (Rugh, 1935). Some caution is desirable, however, in assuming a necessary similarity in behaviour, consequent upon similarity of origin. In the part of the coelom which becomes nipped off as the myocoele, i.e. the cavity of the somite, there is a clear indication that the external boundary wall develops quite differently from the internal wall, and the muscles which develop from these two walls may do so by different methods (Kerr, 1919). In such a situation there is the probability that different "inducing agents" act on the opposite sides of the myotome. There is also the possibility that since the myocoele cavity is a fluid-filled cavity, its dimensions may be controlled, as suggested in regard to the blastula, by cells of differing types opposing each other. Indeed, the rather columnar character of the cells on the neural side of the myotome of dipnoan fish (Kerr, 1919), for example, suggests that they may be somewhat different from the more cubical cells of the external wall. In other animals the cells of the myotome may be differently arranged, but there is generally evidence for a differentiation among them. Perhaps therefore one should bear in mind that different parts of the coelomic wall may have different proportions of, or different tendencies to produce, mechanoblasts and amoeboblasts. Attention has already been called to the frequent occurrence of ciliated cells in the coelomic wall, and also to the observation that Chelonia may have their coelomic cavities lined with columnar and phagocytic cells (Delaney, 1929). A possible explanation of ovarian and testicular development might perhaps be considered on the following lines. If, as has been suggested, the ciliated type of cell is one which, in general, is forcing a current of water outwards from itself on the ciliated side and absorbing salt from the external medium on the same side, then the secondary (cortical) cords, if they carried on this type of function when removed from the surface, and with no duct connecting them to the exterior, would be expected to behave rather as the proximal tubules of the kidney may do in tissue culture and form vesicles filled with fluid. Any other type of cell actually enclosed among these cells (not in the fluid caused by their activity) would be expected to lie in an environment rather more concentrated than normal (because of the removal of water) and this might induce or favour the amoeboid or rounded form of cell behaviour. The formation of the ovarian follicles could in this way receive some sort of causal explanation. The "nurse" cells of the primordial follicle try to form a lumen and succeed in forming a follicle. When ovaries are grown in tissue culture, follicular development stops here (Martinovitch, 1938) but, in the body, the gonadotrophins act either on the cells of the membrana granulosa, or, more probably, indirectly on them through the cells of the theca interna (Schmidt, 1942; Hisaw, 1947), and the follicle grows and gradually distends by the passage of fluid inwards. The ovum remains embedded in the wall and is thus surrounded by cells which are maintaining it in a "concentrated" environment.

In the testis, on the other hand, the tubes form a connexion with the mesonephric duct and so the seminiferous tubes become patent.

The Sertoli cells, i.e. the cells lining the seminiferous tube, fill the tube with a dilute fluid and thus provide the environment for the germ cells, which are here not completely surrounded by the Sertoli cells as was the ovum by the granulosa cells. In this medium the germ cells assume the flagellate form and develop into spermatozoa. This would suggest that the form of the male or female gamete may largely depend, as *Naegleria* does, on the concentration of salts or cations in its immediate environment.

This difference in position and surroundings may seem a very trivial difference to be the cause of the differentiation into male or female germ cells, and this is probably a very much oversimplified picture, yet it is indeed known that the development of an indifferent gonad into ovary or testis often appears to depend upon some quite small stimulus.

In cyclostomes the ovaries may develop from the anterior portions of the gonads while testes arise posteriorly; in other animals the reverse may occur.

In the cyclostomes and the teleosts the medullary component of the gonads does not develop and the entire stroma is probably comparable with the cortex in other animals (d'Ancona, 1952). The different spatial distribution of the male and female germ cells in the animal as seen in the cyclostomes is therefore perhaps to be considered as another method for providing a different environment for the male and female germ cells. Similarly in some teleost fish (e.g.Acanthopterygia) the female cells remain in the gonadal cavity, while the male germ cells become embedded in the stroma (D'Ancona, 1952). In some elasmobranchs, whether the indifferent gonad becomes ovary or testis depends on whether the medulla or cortical part survives. Chieffi (1954) has observed that testosterone, oestradiol, progesterone and deoxycorticosterone all favour the production of cortical tissue and suppress the medulla, and the sexual state can thus be altered. As in so much of this type of work, however, it is not known whether such actions are direct or result from a complex hormonal interplay. In amphibia much the same is the case. Frogs, genetically destined to become males, pass through a stage when they have ovaries, and the cortical parts only degenerate after the medullary parts (the testis) establish contact with the mesonephric rudiments, and this formation of an open tubular system may be an important factor in determining the nature of the differentiation. It is, therefore, again important to decide what causes one part to develop in preference to another and what is the nature of the difference between cortex and medulla. In birds Willier *et. al.* (1937) have shown that small doses of oestrogens cause the undifferentiated gonad of a genetically determined male to become female, while male hormones cause the potentially female gonad to become ovotestis.

The dominant factor in determining the course taken by the germ cells could be very similar to that which determines the behaviour of the cells in the primitive blastula, e.g. the exact ionic conditions in their particular environment, and the manner in which the cells can respond to it, by virtue of the action of pervading hormones and metabolites. If the "coelomoducts" are considered to be regulating tubes displaying a gradient system, and we have seen that there is evidence for such an hypothesis, then the behaviour of a germ cell housed in the tube may well depend on its exact position in the gradient. Moreover, the general ionic condition of the body may alter the setting of the ionic balance within the tube from time to time. A third factor that may be of importance, is the exact position occupied by the germ cell relative to its nurse cells. This point may be illustrated by reference to *Amphioxus* in which the gonad arrangements are far more simple and in which the cells of the general body cavity specialize directly to form the "nurse" cells, and there is little indication of any gradient system (Fig. 14.8). The germ cells become housed in follicles attached to the segmental septa, and in each case a cavity develops among the clusters of cells forming each gonad rudiment. In the male the cavity is central and lined with both germ-cells and "supporting" cells, more or less alternating in position. In the female the cavity is eccentric and lined entirely with "supporting" cells so that the germ cells are removed from the cavity by a "screen" of supporting cells (Zarnik, 1904). The situation in this respect, therefore, is exactly comparable to that in the ovaries and testes of the higher vertebrates, where the male germ cells and Sertoli cells both keep contact with the lumen of the seminiferous tubule, but in the ovarian follicle the ovum is separated from the follicular fluid by the layer of supporting cells that forms the corona radiata (Fig. 14.9). The follicular fluid which is produced by the granulosa cells, with perhaps at a later stage some additions from the theca interna cells, can thus be considered as the fluid formed by the secretory activity of the cells at the coelomic end of the coelomoduct, again with some later additions from cells further along the tube. The pronephric funnels and their homologues, of which the follicles are probably one, are often composed of flagellate cells, and it is therefore interesting to find that follicular fluid, as obtained from cows (after injection of pregnant mare serum (P.M.S. in order to enlarge the follicles), has a low cation content (with low

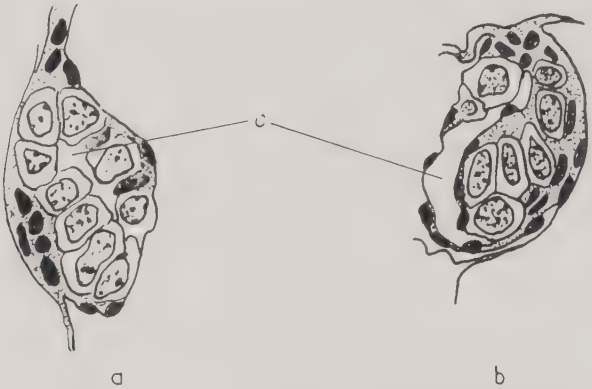

FIG. 14.8
Developing testis (a) and ovary (b) of *Amphioxus* showing the relative positions of germ cells and nurse cells. (c) is the primary cavity, which in the ovary is almost completely lined with nurse cells. (After Zarnik, 1904).

sodium and high potassium (as compared with plasma (Lutwak-Mann, 1954) and it also contains an appreciable quantity of hexosamine. If these figures (Table 14.1) can be regarded as significantly different from those for the circulating blood, they suggest that these cells are secreting a cation-deficient fluid. The P.M.S., of course, may have altered the figures from those which might apply to the fluid produced by the granulosa cells alone, for there is evidence that the enlargement of the follicle which is dependent on pituitary hormones may be at least partly caused by the theca interna cells, which are relatively inactive till stimulated by the pituitary gonadotrophins. If, then, the granulosa cells are producing this cation-deficient fluid—which incidentally also contains some mucoproteins and oestrogens—it is pertinent to consider what is the nature of the environment to which the ovum itself is subjected, lying as it does at the opposite pole of the secretory cells from the follicular fluid. Does it mean that the ovum lies in an environment more concentrated than normal?

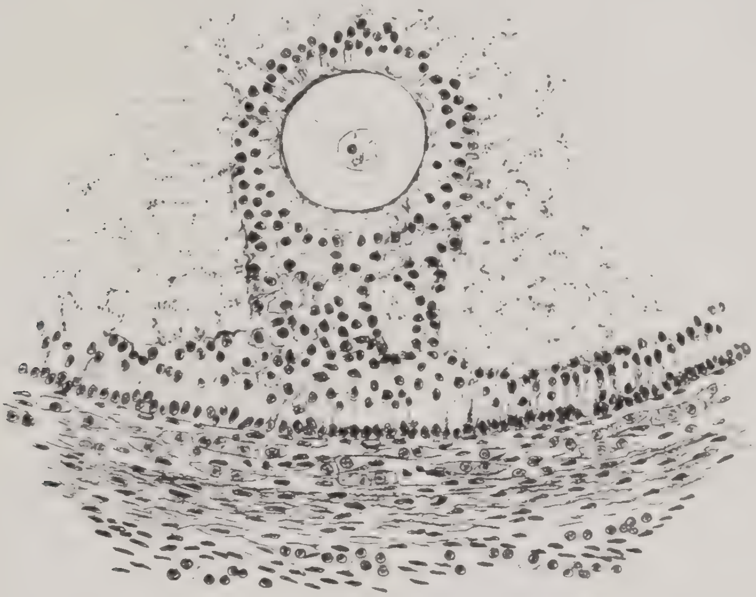

FIG. 14.9

Ovum surrounded by corona radiata cells and thus separated from the follicular fluid. Groups of theca interna cells can be seen between the theca externa and the orientated cells of the membrana granulosa.

By contrast, the male germ cells mature in a tube lined by other germ cells and by Sertoli cells. The contents of the tube are again dilute and exceptionally poor in chlorides and glucose, but, in this case, the maturing germ cells are exposed to the dilute fluid and it is tempting to think of them as being subjected to the same sorts of influence as those which cause *Naegleria* to acquire its flagellum. Some figures given by Sørensen and Anderson (1956) for the ionic content of the fluid in the epididymis of the bull indicate a very low content of Na^+ and a very high ratio of K^+/Na^+ (1:1). In view of the relationship between the nature of the cell surface and the ionic conditions prevailing in and around the cell, it is pertinent to draw attention to the assumption of the flagellate form by the developing spermatozoa, and also to the detailed structure of the oocyte which, in the frog, for example, has its surface increased to some thirty-five times that of a sphere of corresponding diameter by the development of innumerable microvilli (Fig. 14.10) (Kemp, 1956).

TABLE 14.1

	Cow Follicular Fluid mE/l	*Cow Serum* mE/l
Na	110	142
K	8·2	4·8
Cl	110	104
Glucose	43 mg/100 cc.	46 mg/100 cc.

The interstitial cells of the gonads, as derivatives of the coelomoducts, must be considered as adept at producing local ionic environments and as themselves probably rather easily adaptable to changed conditions, i.e. in a state not unlike *Naegleria* and the cells of the primitive blastulae. When the gonads develop, they do so from cells endowed with the capacity for ionic regulation and with a tendency to form tubes with ionic gradients along them. Probably, however, the cells should be regarded as exceptionally plastic and ready to respond to changed conditions, i.e. not yet rigidly determined towards amoeboblast or mechanoblast.

If this is the correct concept of the interstitial cells of the gonad, then the exact homologues with particular parts of nephridia or coelomoduct elements become of far less significance; though whether the cells were originally derived from nephridial or coelomoduct systems, or from portions of the original coelomic cavity, may have left its mark and still be important. The behaviour of each cell and the manner in which it is responding to its immediate local environment become of much greater interest. Since the concept of the gonads being derived from segmental tubes, with a gradient of activity forced on them by virtue of their regulating action, is probably basically correct, traces of this tubular behaviour and its consequent gradients must be expected to persist, though often overlaid with later specializations, which may obscure the primary pattern. For this reason, a study of the polarities of the cells and of the peculiarities of their metabolism and cytochemistry, and of the nature of the other subsidiary activities going on in different parts of the urinogenital system would certainly be rewarding in establishing the true homologues. Once these are established, new comparisons could then be made and some of the specific peculiarities better understood. For example, the spermatic tubules in the mouse, in order to find their exit, join end to end with tubes which evaginate as outgrowths from the

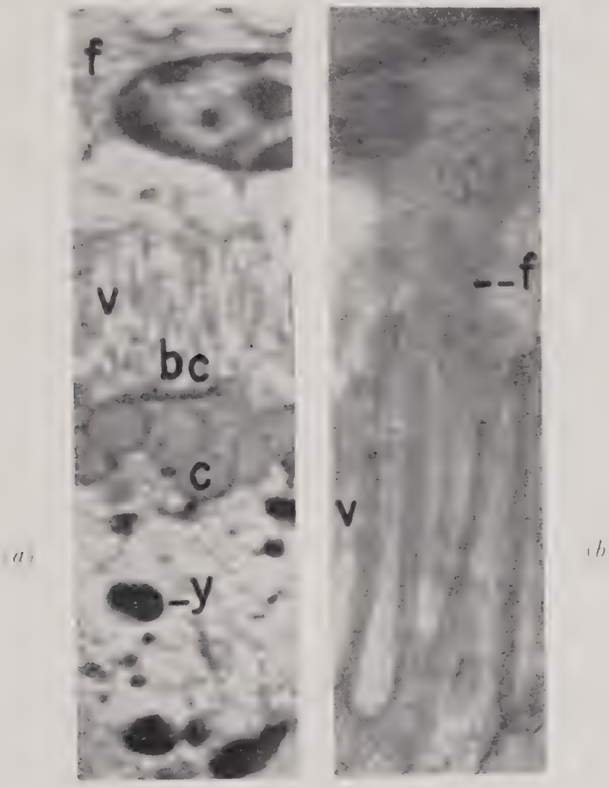

Fig. 14.10

(*a*) Microvilli on the surface of the frog's ovum as seen with the electron-microscope. bc, basal cortical cytoplasm; c, cortical granules; f, follicle cell; v, microvilli; y, yolk. (*b*) The villi at higher magnification (Kemp, 1956).

tubules which leave the Bowman's capsules of the mesonephros, and which in the other direction establish openings (nephrostomes) with the coelomic cavity. What significance is to be attached to these tubes? Are they a specialization in the mouse or were they derived from some pre-existent structures? Something similar is also found in other species, e.g. the opossum (Morgan, 1943) and in other marsupials (Fraser, 1918). Are they remains of connexions between the main coelomic cavity and portions of the cavity nipped off as Bowman's capsules? Could the spermatic tubules be remains of true coelomoducts while the mesonephric elements are nephridial in origin, the whole system thus constituting a mixonephridium; or are both spermatic tubes and mesonephric elements variations on the coelomoduct theme? In this latter case, the end to end junction might have the same significance as the secondary multiplication of the nephric elements in the cortex of the metanephros. The origin of the rete testis and rete ovarii is, from this point of view, a very important problem, and the evidence is unfortunately conflicting. Whereas in some species, e.g. elasmobranchs (Parker and Burland, 1909) and mouse (Brambell, 1928), mesonephric elements seem to contribute to the rete, in experiments on tissues from the albino rat the rete tissue develops in grafts of rudiments of testis or ovary growing in the anterior chamber of the eye, while mesonephric rudiments do not develop such tissue (Torrey, 1947).

An end-to-end junction almost certainly takes place between gonadal and nephric elements, but at this stage there is little to suggest whether the partners in these fusions are identical, and Nature has reaped some advantage from a repeat pattern, or whether the two partners are somewhat different in kind and she is making use of both. Moreover, as has been stressed in the case of the kidney, the actual direction of growth of a tubular structure in embryology may be quite other than its original direction, and thus may not help in determining the origin of the structure.

Before penetrating further into this aspect of the problem, it will be advantageous to digress for a time in order to consider the development and properties of the adrenal cortex, for this is a tissue which in the adult mammal shows very little general similarity with either kidney or gonad, yet which developmentally, phylogenetically and physiologically has much in common with both. A general and comparative study of the cells of kidneys, gonads, and adrenals can then be undertaken.

Meanwhile, it may be tentatively concluded that the supporting and interstitial cells of the sex glands in mammals, and indeed in most vertebrates, are certainly derivatives from coelomic epithelium and probably from a "coelomoduct" system which, in the male, becomes more or less intimately associated with the tubular system of the mesonephros (Fig. 14.11). It is thus probable that these cells are more related to "true" coelomoduct cells than to cells having an originally nephridial origin. They would thus appear to be related on the one hand to the cells of the proximal convoluted tubules of the kidney; and on the other to the special part of the coelomic epithelium from which they are derived; more particularly, the follicle cells compare with the cells of Bowman's capsule and of the neck tubes, and the Sertoli cells and theca interna cells (?) with those further along the tube. The sex-cords thus ideally present a gradient along their length, not unlike that shown in the proximal tubes, though this may naturally be greatly modified in so far as the invasion of the mesenchyme by the coelomic epithelium does not necessarily take the form of definite cords or tubes or persist as such; the follicle cells of the ovary are derived from cells high in the gradient, while the cells of the sex cords which break up to form stroma cells and the cells of the theca interna, are probably cells from the lower and more strongly athrocytic part of the

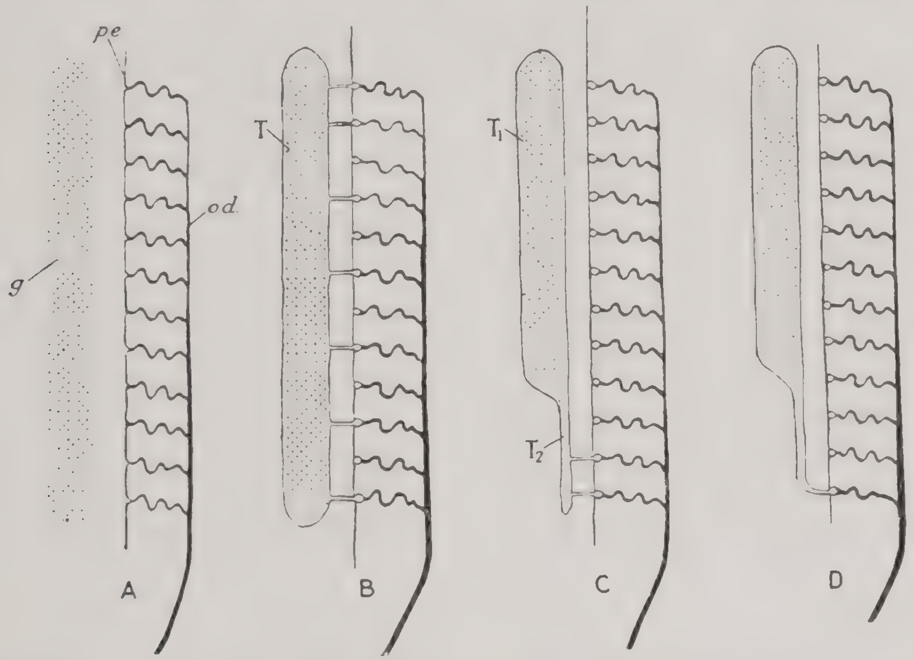

Fig. 14.11

Diagram of the essential patterns of the urinogenital systems in B. *Lepidosteus* and *Acipenser*, C. *Lepidosiren* and D. *Protopterus*, showing the general relationship between the gonads and the nephric units. A represents a hypothetical primitive condition. (Kerr, 1919).

gradient. In the testis the cells corresponding to the granulosa cells of the ovary probably do not persist, the cells of the tubules i.e. the Sertoli cells, are probably in much the same position on the gradient as the theca interna cells of the ovary, while the interstitial cells behave as if they were derived from a position lower in the gradient. They are often very strongly athrocytic. On the other hand, it has been reported (Esaki, 1928) that, in tissue culture of cryptorchid testes, interstitial cells and Sertoli cells both become spindle-shaped elements corresponding to mesenchymal (mechanocyte) cells, and that other cells, described as spermatogonia, store carmine and may appear as multinucleate giant cells, rather in the manner of amoebocytes. Further investigation would seem to be desirable.

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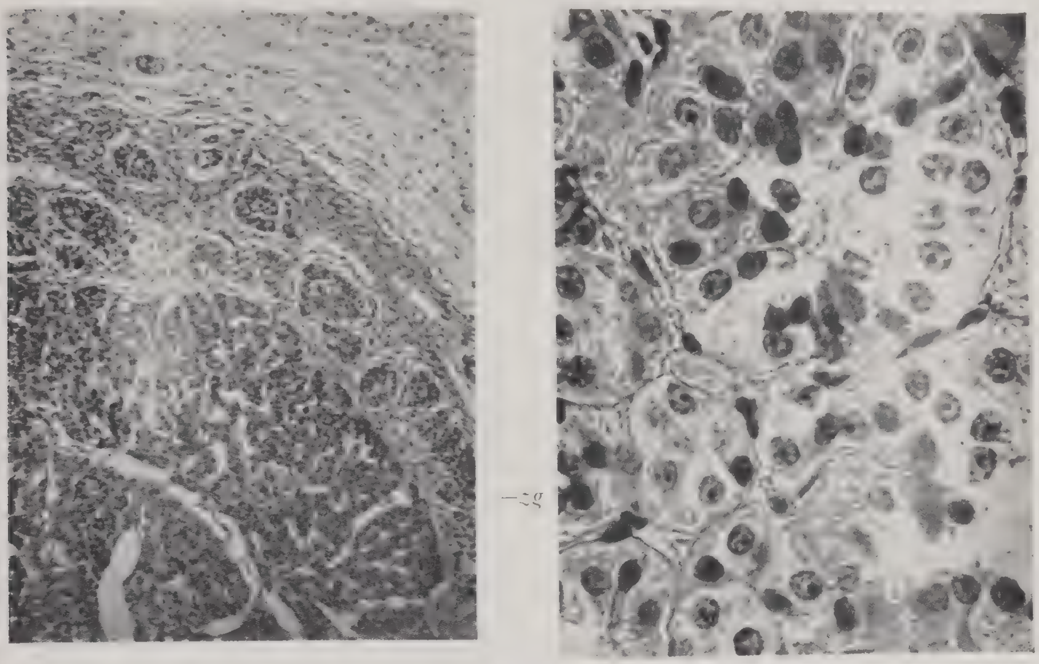
Chapter 15

COELOMODUCTS, NEPHRIDIA AND ADRENAL CORTEX

The adrenal cortex, which is homologous with the inter-renal tissue of lower forms, arises embryologically in mammals and, indeed, in most vertebrates in much the same way as the genital ridge, of which in fact it may form a part, by a thickening of the dorsal coelomic epithelium. In this region there is no basement membrane and an ingrowth of the epithelial cells into the underlying mesoderm gives rise to cord-like arrangements of cells. In man, Uotila (1940) has described two such invasions of cells, the first of acidophil cells forming the so-called foetal cortex which ultimately degenerates, and the second to form the permanent cortex of more basophil cells. The nature of the cells taking part in these invaginations is still problematical but the comparison between this situation in the embryonic adrenals and the medullary and cortical invaginations in the gonads is perhaps apposite. Interestingly enough, there are often present among the cells of these cords large round cells (Hett, 1925; Uotila, 1940) comparable in appearance with the germ cells in the similar gonadic cords. In the rudiment of the adrenal cortex, these cells usually degenerate but their early presence emphasizes the close relationship between adrenal cortex and gonadic structures. This presumably means that the adrenal cortex must be included in any consideration of the more recent history of the "coelomoducts".

As development proceeds, in mammals, the cords of cells forming the permanent cortex elongate and differentiate into two rather definite zones, the zona fasciculata and the zona glomerulosa; the first of these is characterized by relatively straight cords of cells, though in some species they may become intertwined, as they are in the reptiles; in the second and more superficially placed zona glomerulosa there is a tendency for the cells to form a more vesicular or follicular pattern. In cattle and horses, actual vesicles appear and, in the former, the zona glomerulosa may be completely separated from the zona fasciculata by a definite layer of connective tissue (Weber, McNutt and Morgan, 1950) in much the same way as the follicular layer of the ovary may sometimes be separated from the sex cords (compare Fig. 15.1 with Fig. 14.6). Even in man there is a stage of development (about 100 - 150 mm embryo) when the zona glomerulosa is composed of vesicles (Hett, 1925). In cattle, the vesicles may not be closed, but their cavity may be invaded by connective tissue and capillaries in a manner not unlike a rudimentary form of renal glomerulus.

The inpushing coelomic epithelial cells may become completely separated off from the coelomic epithelium itself by a layer of connective tissue, just as they do in the testes by the formation of a tunica albuginea. Alternatively, continuity with the coelomic epithelium may be maintained and cells may continue to penetrate from the epithelium to join the zona glomerulosa (Gruenwald and Konikov, 1944; Baxter, 1946; Gruenwald, 1942a, 1946). This continued recruitment has been observed in several species just as similar cells continue to penetrate into the ovaries of many species either as Pfluger's egg tubes or as new follicles (Witschi, 1951).



(*a*) (*b*)

FIG. 15.1.
The zona glomerulosa of the adrenal cortex in cattle.
(*a*) c, cortex; m, medulla; zg, zona glomerulosa; (*b*) Vesicles in the zona glomerulosa (Weber, McNutt and Morgan, 1950).

In the mammalian adrenal cortex, a third layer of cells, the zona reticulata, persists or develops centrally; this corresponds in general structure and position with the rete ovarii and rete testis and may well have much the same origin as these structures, i.e., it may correspond with nephrostomes of the mesonephros (Gruenwald, 1942b) (Fig. 15.2). In some animals, such as the mouse, there is also another zone known as the X-zone distinct from the zona reticulata, and, like the foetal cortex in man and also the rete ovarii, it sooner or later degenerates (Deanesley, 1928). The X-zone is generally considered to be quite distinct from the zona reticulata, though it may have something in common with the human foetal cortex. In the male mouse it degenerates at puberty; in the female it regresses slightly with each sexual cycle and completely in pregnancy, suggesting that it is suppressed by the action of circulating male hormone or of progesterone-like hormones (Fig. 15.3). The pituitary gonadotrophins seem to be responsible for its growth, perhaps indicating that it is composed of tissue comparable with the cells of Leydig in the testis.

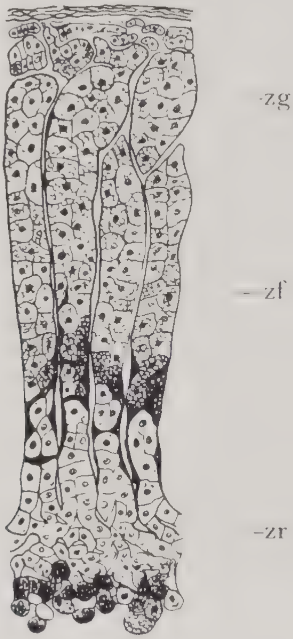


Fig. 15.2.
The cellular arrangement in the mammalian adrenal cortex.
zg, zona glomerulosa; zf, zona fasciculata; zr, zona reticulata.
(Cowdry, 1938)

From the developmental point of view, therefore, there is a very obvious similarity between the adrenal cortex and the interstitial and accessory cells of the ovary and testis. Their common origin from the coelomic epithelium and their close connexion with nephrostome-like structures corresponding to the Wolffian body emphasize that physiological similarities are to be expected between the two groups of tissues and, at the same time, raise questions as to the nature of the cells which enter into the complexes so formed. Are the adrenal cortical cells to be regarded as modifications of pre-existing coelomoducts, and/or nephridia, or merely as modifications of the coelomic epithelium itself in a new direction? The cord-like arrangement certainly suggests tubes—but the question of what tubes remains to be answered.

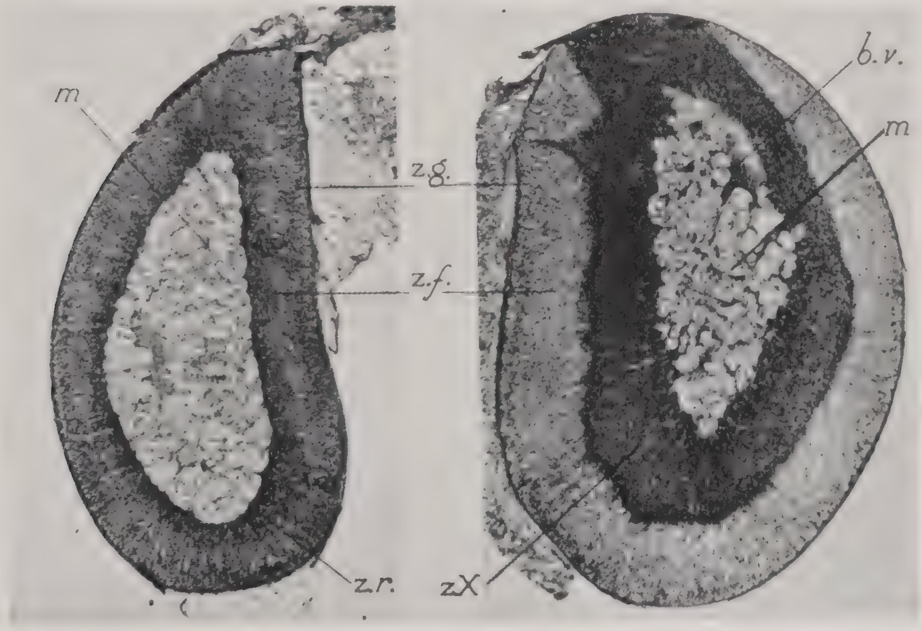


FIG. 15.3.
The X-zone in the adrenal cortex of the mouse.
(*a*) 12 weeks old male: X-zone absent. (*b*) 12 weeks old unmated female: large X zone. bv, blood vessel; m, medulla; zf, zona fasciculata; zg, zona glomerulosa; zr, zona reticulata; zX, X-zone. (Deanesley, 1928).

In the reptilia, the cords of cells are very well marked and are enclosed in connective tissue capsules in a manner reminiscent of the seminiferous tubules of the testis, except that the cortical cords remain solid. In *Natrix,* however, the cords may, under the influence of adrenocorticotrophic hormone of the pituitary (ACTH) which in most mammals causes hypertrophy of the zona fasciculata, show degeneration of the central cells and may eventually hollow out to form tubular structures (Wright and Chester Jones, 1957). In nearly all species of mammals tested, the hypophysis, by the action of ACTH, appears to maintain the zona fasciculata in a state of functional activity, but the zona glomerulosa is not usually so dependent. In fact, on these and other grounds, there are strong reasons for considering the zona glomerulosa and zona fasciculata to be functionally different, and the evidence is steadily accumulating in favour of the view that the zona glomerulosa is the source of aldosterone and the mineralocorticoids (Stoerk, Knowlton and Loeb, 1955; Giroud, Stachenko and Venning, 1956), while the fasciculata is more concerned with the production of the glucocorticoids, though in most species this is probably an over-simplification and the distinction between mineralocorticoids and glucocorticoids is not absolutely sharp either with respect to their seat of origin or their physiological action. Treatment of mice with deoxycorticosterone (DOCA) causes atrophic changes in the zona glomerulosa cells and so also does increasing the ratio Na^+/K^+ in the blood (Miller, 1950). The other layers are relatively unchanged.

Hypophysectomy has been found to reduce the output of steroids into the blood flowing through the adrenal to about 10% of their normal level, with the exception of aldosterone, which is only reduced to about 40%; the subsequent addition of adrenocorticotrophin (ACTH) to the blood increases the output of all steroids except aldosterone (Farrell, Rauschkolb and Royce, 1955). These observations taken in conjunction with those on the production of the appropriate steroids by tissue slices composed primarily of either zona glomerolosa or fasciculata (Ayres, Gould, Simpson and Tait, 1956), and with the observed histological changes in the adrenal cortex in response to hypophysectomy and treatment with ACTH, point strongly towards this division of labour among the cells of the cortex.

Some very interesting light is thrown on the difference between glomerulosa cells and fasciculata cells by their reaction to being placed as homografts in chambers made in the rabbit's ear. Under these conditions the cells of the zona fasciculata undergo a progressive atrophy and they are not usually vascularized. On the other hand the zona glomerulosa cells stimulate a very rapid vascular reaction and become immediately invaded by a network of vessels (Williams, 1945). Indeed, when two grafts are made close together, the veins draining from the first may provide a "portal" supply to the second. This vascular stimulation by the zona glomerulosa will be seen later to have some further implications. The vascularity of ovarian follicles and of glomeruli in the kidney immediately invites comparison.

In the embryological development of amphibia, the adrenal cortical cells first appear as an unpaired organ between the aorta and the vena cava. In this, curiously enough, they resemble the gonadal rudiments in the lamprey (Wheeler, 1899). The cells then separate and travel laterally and along the ventral surface of the mesonephros, thus again emphasizing the close association between these tissues. Treatment of tadpoles with oestradiol has been found to cause great hypertrophy of the adrenal tissue, which remained as a large unpaired organ, and the enlargement appeared to take place at the expense of the mesonephric elements, as though these tissues could, by alteration in the local conditions, be switched from one form of differentiation to another, i.e., to produce kidney, adrenal cortex, or gonadal tissue (Segal, 1953).

Amphibian adrenal tissue is interesting in two other ways; first, it is extremely variable throughout the seasons of the year and in relation to the sexual activity of the animal, and in many cases it is intimately connected with the varying salt and water balance of the animal. It is significant, for example, that adrenalectomized summer frogs can be kept in perfect health in isotonic salt solution (Chester Jones, 1957) though not in solutions necessitating active regulation of the animal's salt content. This clearly points to the adrenal gland as playing a dominant part in regulating the salt content of the animal. Secondly, as in the adrenals of some reptilia, birds and mammals, the cortical cells may be heavily loaded with carotenoid pigments, which again vary with the season and the state of sexual activity. This property is, of course, in many animals shared with ovarian cells, especially those of the corpus luteum, and with the interstitial cells of the testis, and may reflect nothing more than the solubility of carotenoid pigments in the particular lipids of these tissues. It is interesting, however, that these pigments are strongly associated with the reproductive organs in a very wide range of organisms, both vertebrate and invertebrate. Even in the adrenal tumours associated with virilism in women, there is often a heavy carotenoid pigmentation, in addition to the liberation of male hormones. These tumours are generally associated with the inner zones of the cortex.

In fish, the adrenal or inter-renal tissue varies greatly in amount and location from being present as scattered cells or groups of cells in the region of the cardinal vein, to forming fairly well-developed organs, some of which, like those of higher vertebrates, respond to hormones from the pituitary. In addition to the tissue that responds in this way, certain fish have other collections of tissue with a more or less vesicle-like structure: these bodies are called the corpuscles of Stannius. They generally develop more posteriorly than the inter-renal tissue and from the archinephric duct rather than the dorsal "mesothelium" (Garrett, 1942). For these reasons they have been regarded as tissue of a different kind. They are well developed in the salmon and in a Mexican fish *Astyanax* (Fig. 15.4). In the latter case, some genuine inter-renal tissue responds to addition or removal of pituitary influence but the corpuscles of Stannius do not, and for this reason also they have been thought not to belong to the inter-renal complex. Nevertheless, their vesicular structure, their presence in fish which can tolerate changes in the salt content of their environment (e.g., the salmon) and their failure to react to hypophysial hormones (as in *Astyanax*) may all be taken as points of similarity to the cells of the zona glomerulosa of higher forms. Like the zona glomerulosa,

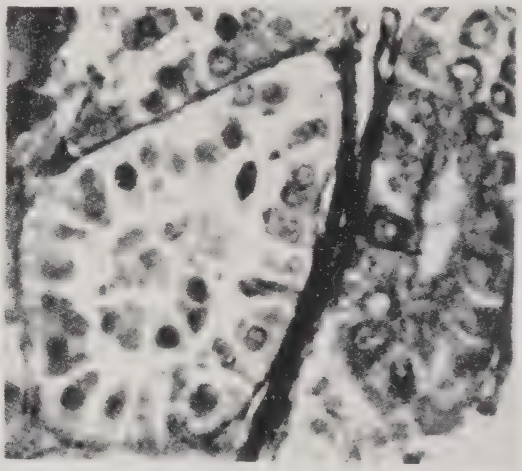


FIG. 15.4.

A vesicle of the corpuscle of Stannius in the Salmon. A kidney tubule is seen on the right (Chester Jones, 1956).

they also contain phospholipid granules (Fig. 15.5). The number of these granules varies with the water load on the fish and decreases when the fish is kept in 1% NaCl (Rasquin, 1956) so that the cells appear to resemble the zona glomerulosa cells on these grounds too. There thus seems no clear justification for not considering them as part of the adrenal complex.

While there is little doubt that coelomic epithelial cells may, in some species, continue to penetrate the capsule of the adrenal and to join the cells of the zona glomerulosa, there does not seem to be much direct evidence for the general idea of progressive cell movement from the outer cortex towards the medulla with a growth zone in the glomerulosa or "intermediate" zone as has been suggested by several authors. Plotting the positions of occurrence of cell divisions in the cortex does not, by any means always, show them to be restricted to the outer zones. On the other hand, degenerating cells are frequently found near the border between cortex and medulla as would be expected on an "escalator" theory (Hoerr, 1931; Bennett, 1940).

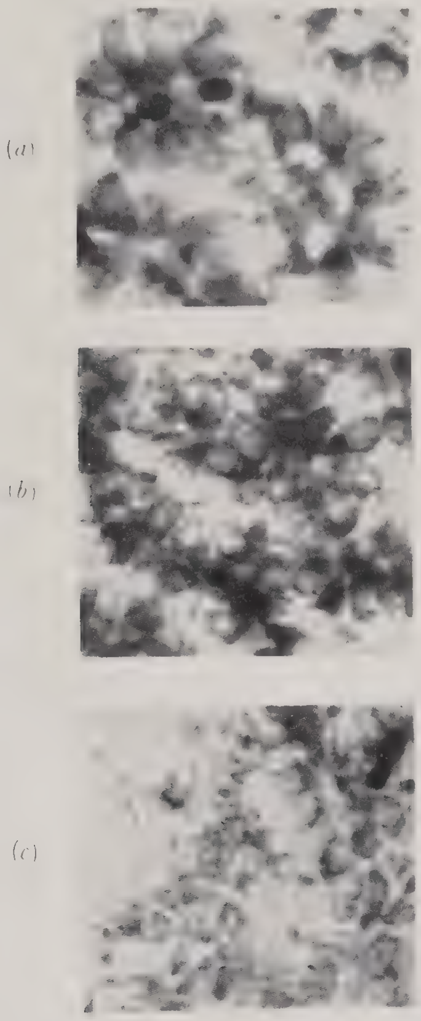


FIG. 15.5.

The corpuscles of Stannius in *Astyanax mexicanus*.

(*a*) Control fish (*b*) From a fish with an increased water load, caused by injections of water. (*c*) From a fish living for 10 days in 1% NaCl The cells are stained with Baker's acid haematein and show phospholipid granules, more in (*b*), but far fewer in (*c*). (Rasquin, 1956).

While such a slow replacement of cells is not impossible, the idea is a little difficult to harmonize with the rather pronounced difference between the cells of the zona glomerulosa and those of the zona fasciculata.

By far the most characteristic cytological feature of the adrenal cortex is the occurrence within its cells of lipoid material, in which phospholipins, cholesterol and other sterols and their esters are often present in large amounts. In the past, the amount of lipid present in the cells has often been taken as a measure of functional activity, but measurements of the hormonal activity of adrenal effluents have shown how erroneous this concept may be. Hormone production may far exceed the amounts that can be extracted at any one time. The cells of the adrenal cortical tissues undoubtedly have a great capacity for storing and metabolizing lipids and, in particular, the steroids. Their activity as hormone producers, however, cannot necessarily be assessed by their content of steroids and still less by their total content of lipids. The variation in the content and distribution of the lipid both from time to time in one animal and from place to place within the cortex of different species makes any simple interpretation at this stage almost impossible. Both the chemical nature and the actual amounts of the lipids present are difficult to estimate cytochemically, though the newer techniques, like the modified Schultze reaction for particular steroids (Lewis and Lobban, 1959), are beginning to assist. Furthermore, the exact causes of the variation which can take place in lipid content by alterations in the hormone balance of the organism cannot easily be assessed. Castration of the rat floods the zona fasciculata of its adrenal with lipid and subsequent treatment with testosterone entirely depletes it again, while the zona glomerulosa remains apparently unaltered throughout. What the lipids are that appear and disappear and what is the nature of the stimulus to the adrenal cells, e.g., testosterone level in the blood, gonadotrophin level in the blood, or some as yet unsuspected hormone, are questions which still remain to be answered. Man has more lipid per cell in his zona fasciculata than in the zona glomerulosa; so has the cat, but not so the rat. There is always the possibility that the production of granules in cells may represent the accumulation of by-products or metabolites rather than the storage of the active secretion.

In addition to the occurrence of lipids and of carotenoid pigments, a character which the cells of the adrenals share with those of the sex glands and kidneys, there are one or two other cytological peculiarities of the cells in different parts of the adrenal cortex which may be of importance in establishing their inter-relationships. For example, the cells that are sometimes to be found in the capsule and that are thought to join the cells of the zona glomerulosa, have the capacity for storing trypan blue and similar dyes in characteristic small granules in a manner rather different from that found in macrophages (Salmon and Zwemer, 1941; Baxter, 1946). This property is not unlike that of the cells of the proximal convoluted tubules of the kidney, and of some of the interstitial cells in the testis (Rasmussen, 1932).

Secondly, it is not uncommon to find cells in the adrenal cortex which exhibit a strong fuchsinophilia. In mammals, these are particularly associated with the inner parts of the cortex and zona reticulata, but in other groups special fuchsinophil cells may be located in other positions also. This fuchsinophilia is often associated with a brown or yellow pigmentation and with an affinity for iron (siderophilia), and, once again, these properties have also been observed in the cells of certain parts of the proximal tubules of the kidney (Grafflin, 1942). Another characteristic feature of the adrenal cortex is its extraordinarily high content of ascorbic acid, which is localized for the most part in the inner zones of the cortex -the deeper zona fasciculata and the zona reticulata. This property it shares with the corpus luteum.

The distribution of phosphatases and esterases in the adrenal cortex of those species which have been examined is by no means uniform and often the reactions of the cells in different zones are very different in different species. For example, the human adrenal gives a positive reaction for alkaline phosphatase in the inner zones of the cortex, while, perversely, the guinea pig shows it most strongly in the outer zones (Gomori, 1941). Similarly a positive pseudocholinesterase reaction is given by the zona glomerulosa of the cat (Koelle, 1950) but not of the rabbit. In the rat, the reaction is negative in this zone, though very strongly positive in the capsule (Coupland and Holmes, 1958). How far these cholinesterase reactions are dependent on the distribution of nerve fibres and how far they are the properties of the cells themselves, is still uncertain. It is perhaps interesting that the rat is a species which shows capsular cells being added to the zona glomerulosa (Baxter, 1946). These cytochemical observations are, in general, too difficult to interpret at the present time and are often very conflicting; this, however, is not surprising since different substrates have been used by different investigators, different species have been examined, and little attempt has been made to correlate the observations with the sexual or other activity of the animal or to standardize the conditions in any way. Much the same criticism makes any interpretation of the cytological effects of various hormones on the adrenal cortex almost meaningless. The cortex certainly changes its activity in relation to changes in sexual activity, hypophysial stimulation, "shock", salt metabolism, anaesthetics and a variety of other conditions (e.g. "stress"!). The most satisfactory ways so far available, in which the direct response of the cortex to individual stimuli can be studied, are by those tissue culture methods which allow the cortex to survive in a differentiated form, i.e., by organ culture. Only by some such means of isolating the organ can the secondary effects of other organs be eliminated, and any direct effects be observed. In such an extremely complex balance as is normally established by the endocrine system of an animal, in order to maintain homoiostasis, it is not surprising to find that different species maintain homoiostasis with different "settings" for the activity of their various regulating organs and such differences may or may not show up in the cytological features of the organs which can be studied by the techniques available. Even with organ culture, the problem is unlikely to be simple, for it may be necessary that, before hormone A can exert its action, the "target organ" shall be primed with hormone B. For example, prolactin only acts on a mammary gland previously "developed" by oestrogens and progesterone. Such priming may be difficult to achieve *in vitro*.

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CHAPTER 16

COELOMODUCTS, NEPHRIDIA, KIDNEYS, GONADS AND ADRENAL CORTEX

IN THE preceding pages attention has been called to the probable similarity in origin of the gonads (other than the germ cells themselves), adrenals and kidneys, not only embryologically, but also phylogenetically. It has been suggested that all three organs are derived from "coelomoducts", using the term in its widest sense, i.e. relating to serially arranged tubes connecting the coelom with the outside. These tubes, whatever their origin, i.e. whether from true coelomoducts or from nephridia, must have had as their main function the regulation of the composition of the fluids on either side of their walls; the activity of nephridial derivatives would be more likely to be set by the composition of the tissue fluids, that of the coelomoducts by the composition of the coelomic fluid or the tubular contents. This distinction, though primary, could of course easily be reversed in evolution as, for example, when the tube ceases to be patent and becomes a cell cord; it may therefore have lost much of its significance and thereby lessened the physiological importance to be attached to the primary distinction between nephridium and coelomoduct. The distinction may nevertheless sometimes be a helpful clue in considering detailed cellular function and subsidiary physiological and biochemical features, and in helping to interpret the process of embryonic development. The pattern of behaviour of certain cell groups may often reflect, more closely than their course of embryonic development, the actual origin of the cells. Thus ectodermal cells pushing inwards from the outside during the later stages of development are likely to differ from coelomic epithelial cells which entered the mesohyl much earlier, and these differences may always remain latent in spite of the similarity in behaviour which may be forced on the cells by virtue of the similar functions which they are later called upon to perform.

Both types of tube, coelomoduct and nephridium, probably always contain as their basic pattern two sorts of cells working in opposite directions on a gradient system, the composition of the fluids, inside or outside, being maintained "constant" or suitably regulated by their opposed actions. These actions might be expected to include the regulation of salts, water and osmotic pressure, pH, and the prevention of the loss of such important constituents to the organism as glucose, amino acids, fatty acids, steroids and other small molecules. It is neither surprising, therefore, that such tubes should become of increasing importance and acquire varied physiological functions as the size of the vertebrate body increased, nor that the kidney, as the primary excretory organ, should be the main result. It will also be important, therefore, to enquire about what happens to those coelomoducts which do not become incorporated in the kidney, namely, those corresponding to the anterior segments of the body and those between the pronephros and mesonephros. Ionic regulation and some degree of excretion could also be carried out by similar tubes modified in other directions also, i.e., not included in the main kidney. It is less immediately obvious why and how the adrenal cortex and the gonadal tissue developed from these same tubes. In the case of the gonads the most likely explanation may be simply that the germ cells became prematurely housed within the exit tubes from the coelom rather than in the coelom itself. If the question is then asked as to what advantage would be gained to the organism in this manoeuvre, then the answer can only be speculative. It may, as already suggested, have resulted from purely structural changes, in the sense that the coelomic cavity as a whole became used for other purposes; for example, facilitating gross bodily movement, or for providing a readily available channel of excretion for the products of the contained organs. Perhaps, as has also been suggested, the germ cells could, by entering the coelomoducts early, be directed into an environment which would facilitate the differentiation of the germ cells themselves, i.e., the ovum could be given a local environment suitable for the development of the round (amoeboid?) storage form of cell, while the male germ cells are given an environment which favours the production of flagella and motility. In view of the change of form of *Naegleria* in this respect, and of the somewhat tenuous threads which hold the balance between the development of male or female gonads in some fish and amphibia, it would seem not unreasonable to suggest that the immediate local environment may sometimes, perhaps always, be a necessary factor in determining the course of the differentiation of the gametes.

The original germ cells, though obviously totipotent, seem usually to be in the amoebocyte form; they develop in sponges from archaeocytes and maintain their amoeboid form for a considerable time: in other embryos also they develop from cells at the vegetal pole. Even in higher animals there are indications that spermatogonia, in tissue cultures of cryptorchid testes, can give rise to giant cells (Esaki, 1928), and this also is a property usually associated more with amoeboblastic behaviour than with that of mechanoblasts. Incidentally, it is interesting to note that under the same conditions the Sertoli cells grow in the manner of mechanocytes. Presumably the germ cells should by regarded as being in much the same labile state as is *Naegleria*, i.e., they are at first plastic and can subsequently assume either form of activity. The observation of Weisel (1948) that the activity of the sperm of a marine teleost is maintained best in diluted sea-water is perhaps pertinent in this connexion, as possibly indicating the influence of ionic concentration on the form and activity of these cells. So also is the suggestion (Schlenk and Kahmann, 1938) that trout sperm acquire motility when their potassium concentration is higher than that of their surroundings. These cells mature in a relatively high potassium concentration (thus resembling the spermatozoa of bulls, see p. 292) and they acquire motility by dilution in water and during their activity they lose potassium. Both total cation and K^+ concentrations are thus likely to be involved in determining first the cell form and then the motility.

In any system involving an epithelial tube there are several local environments to consider and each may have its own equilibrium conditions. If the cells of the tube are concerned with the regulation of the composition of the contents of the tube, they must do so by adding or subtracting constituents respectively to or from those contents. These substances, precursors for them, or by-products from them must, *vice versa*, appear in the tissue fluid and probably in the blood stream on the other side of the wall of the tube or must disappear from these situations according to the direction of the process. Even such an apparently straight-forward function as the redistribution of Na^+ may involve the movement or production of quite complex molecules (c.f. Table 11, 1). These molecules may themselves have important physiological actions which, at some later evolutionary stage, prove to be more important to the organism than the original process of which they were the by-products. So it is that the tissue may, in evolution, become modified for the greater production of the by-product, and by then the original cause of its production may have sunk into insignificance.

For example, reasons have been put forward to support the idea that the cells of Bowman's capsules, the "neck" part of the proximal tubules of the kidney, and the granulosa cells of the ovarian follicle, may have been derived from closely related cells whose activities were in some ways comparable with those of *Naegleria* in the flagellate state, namely, their tendency is to absorb salts from the lumen which they enclose or to add water to it. In the kidney the first activity predominates, the water now being provided by filtration from the blood; in the ovary, the secretion of a dilute fluid by the granulosa cells as the follicle ripens under the influence of pituitary hormones seems a not unreasonable description of the process of ovulation and is to some extent supported by the observations of Lutwak-Mann on the relatively low sodium content of follicular fluid. Moreover, in view of the action of the mineralocorticoids associated with the zona glomerulosa of the adrenal gland in affecting the Na^+/K^+ balance in the body, the different values of this ratio in the follicular fluid (1.3) as compared with the plasma (3.9) is perhaps of some interest (Lutwak-Mann, 1954). The absence of chlorides in the seminal fluid until the seminal vesicles have made their contribution suggests that the fluid in the seminiferous tubules is also likely to be dilute so far as ions are concerned. In this case, a slightly different phase of the activities of the coelomoduct cells predominates, and no doubt other important activities are simultaneously involved. Nevertheless, certain common features remain. The theca interna and the Sertoli cells both produce oestrogens (Huggins and Moulder, 1945; Scott and Lynch, 1952) and, in the cat at least, there is histochemical evidence that oestrogens may also be present in the proximal convoluted tubules of the kidney (Lobban, 1955, 1956). It is not known whether they are produced there, are segregated there and stored, or are merely being excreted, but the amount which is present certainly varies with the amount of gonadotrophins circulating in the blood, and these kidney cells show more "affinity" for the steroids than do most other tissues in the body. Probably the appearance of lipoid droplets here is a phenomenon which is exactly comparable with their appearance in the zona fasciculata layer of the rat's adrenal cortex (see p. 306): for example, both are increased by castration. The other tissue which might also be involved in this complex with the ovarian follicles, and the glomerular capsules of the kidney is the zona glomerulosa of the adrenal and its tendency to produce follicle-like systems, as in cattle (Weber, McNutt and Morgan, 1950), and in the human embryo (at about 100-150 mm length) (Hett, 1925) is perhaps an indication of the capacity of its cells to move fluid preferentially towards a potential lumen. Another straw blowing in the same wind is the observation (Trowell, 1958) that when the rat's kidney tissue is cultured *in vitro* as "organ cultures" in oxygen, the glomeruli after a few days undergo a curious morphological change and become converted into vesicle-like structures surrounded by a double layer of cuboidal or semi-cuboidal epithelium (Fig. 16.1). The contents of the vesicle appear to be disintegrated cells, etc., presumably the results of the breakdown of the vascular part of the glomerulus, but the general behaviour of these cells under these conditions of tissue culture is reminiscent both of ovarian cortical tissue, particularly after the germ cells have been damaged by X-rays (Brambell, Parkes and Fielding, 1927) (Fig. 16.2) and of certain types of adrenal zona glomerulosa tissue (see Fig. 15.1). On the same lines, treatment of

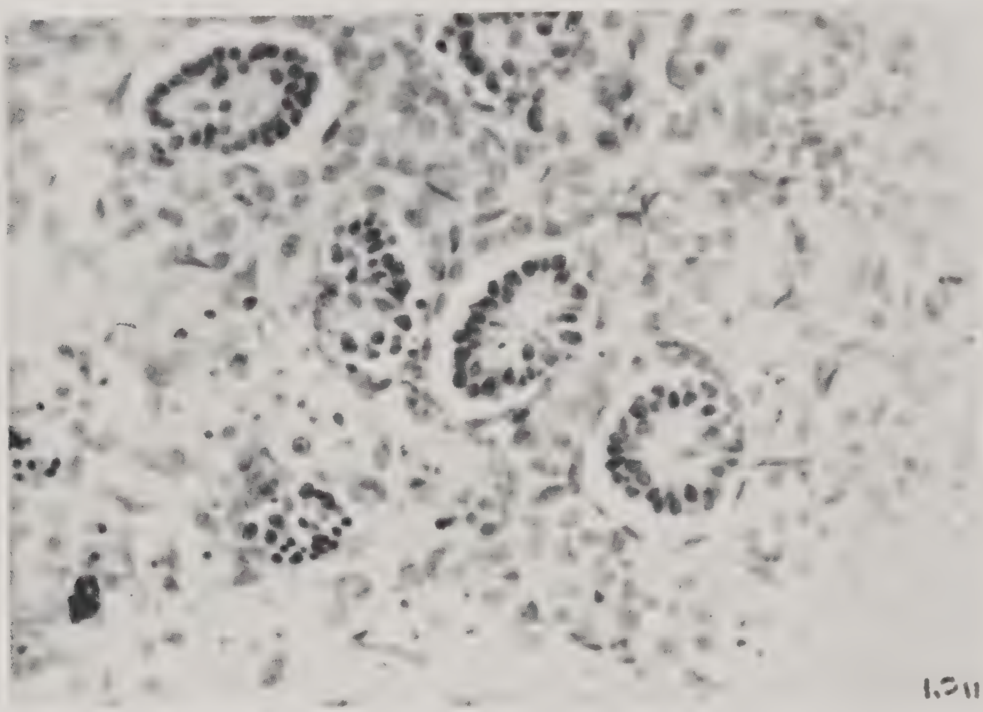


Fig. 16.1.
Changes in the cellular arrangement of the glomeruli of the kidney (rat) which occur during 'organ culture' *in vitro*. (Trowell, 1958).

mice with male hormones also induces the cells in the outer membrane of Bowman's capsules in the kidney to assume a cuboidal form, but the direct cause of this change is not known (Selye, 1939). The glomeruli of the kidney in tissue culture also resemble, in their cellular arrangement, the corpuscles of Stannius (see Fig. 15.4) which occur near the kidneys of salmonid fish and may represent some modification of inter-renal or zona glomerulosa tissue perhaps connected with the salt-balance of these fish, which can adapt to very wide ranges of salinity (Rasquin, 1956) (see p. 303). These organs, unlike the rest of the inter-renal tissue, are, as previously mentioned, not sensitive to ACTH and in this way resemble glomerulosa cells; moreover, they appear to develop from outgrowths of the pronephric duct and so could correspond to nephrostomes and therefore to the glomerular part of the kidney, zona glomerulosa and ovarian follicles. The extreme ease with which the zona glomerulosa cells become richly vascularized in grafts of adrenal cortex inserted into transparent chambers in the rabbit's ear (Williams, 1945) is perhaps connected in some way with the vascularity of the coelomic epithelium near the nephrostomes, which is instrumental in forming glomeruli. It may perhaps be significant to this problem that, if the blood flow to the glomerulosa cells in the grafts is occluded, the cells of this zone become bathed in quantities of fluid. It would be of interest to know how and why this "oedema fluid" arises. The rich vascularity of the theca interna of the ovarian follicle is also to be noted. Indeed, the common feature of these three groups of cells, beyond the fact that they take origin from "coelomoduct" tissue, is that they are probably all derived from that part of the "coelomoduct" lying nearest to the coelomic epithelium, i.e., from the part that often manifests itself as a ciliated funnel and, by analogy with *Naegleria*, may well be the part of the tube most adapted to water elimination and salt retention. They thus form the

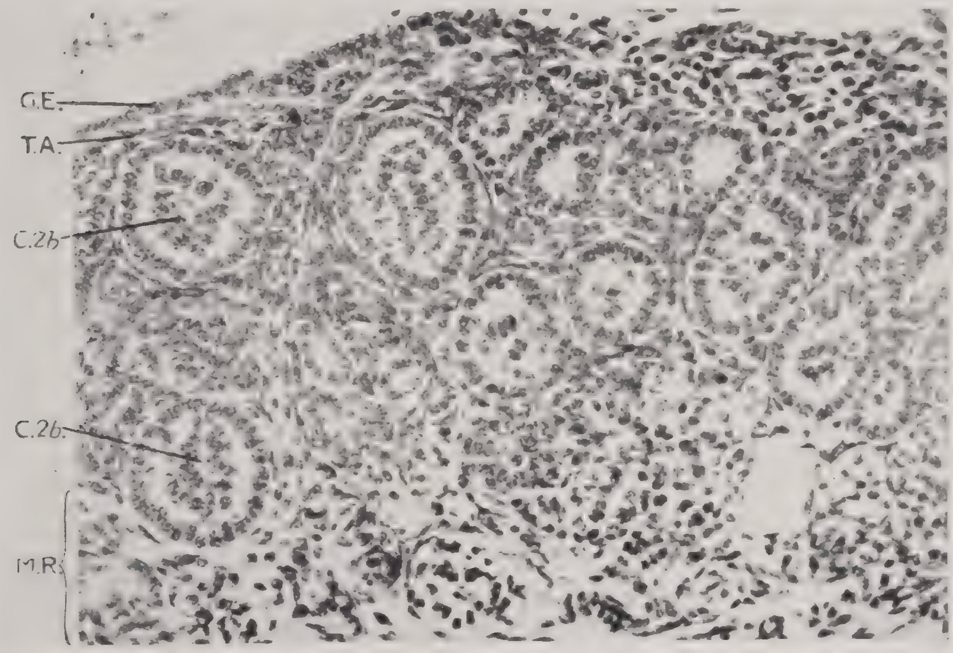


Fig. 16.2.

Cortex of the mouse ovary after the germ cells have been damaged by X-rays. (Brambell, Parkes and Fielding, 1927).

apical point of the gradient. In this way the cells may perhaps be compared to the flagellate cells of a blastula, and the presence of hexosamine (Lutwak-Mann, 1954) and mucopolysaccharide (i.e., metachromatic or PAS-positive material) in the follicular fluid (Wislocki, Bunting and Dempsey, 1947; Halmi and Davies, 1953), just as it appears in blastocoele fluid, is a relevant observation.

Gradients in the "Coelomoducts"

If the original idea is correct that a coelomoduct or nephridium has essentially two sections, a salt-conserving, water-eliminating and a salt-eliminating, water-conserving section, to which of course other specific properties like glucose absorption may be added, then it is reasonable to suppose that the same broad subdivision may still be present in derivatives of these tubes. According to the conditions reigning in any particular animal, the tube may have differing proportions of these two types of cell, or the extent of their activity may vary, and this too may be reflected in the derivatives of the tubes. If, for example, the adrenal cortex as a whole is regarded as being derived from coelomoducts and the zona glomerulosa as corresponding to their ciliated funnels, with water elimination and salt retention as primary functions, then the zona fasciculata might presumably represent those cells of the original coelomoduct which were responsible for extraction of sugar from the tubular fluid, and possibly with salt elimination also. Furthermore, just as in sponge embryos the flagellate cells of the animal pole are sometimes sharply divided off from the posterior amoeboid cells, and are sometimes separated from them by a zone of intermediate cells, so too it might be expected that there would sometimes be a transitional zone of greater or less extent separating the main regions of the cords (nés tubules) in the adrenal cortex. The sudanophobic or "intermediate zone" of the adrenal cortex may well represent just that condition. In addition, on purely logical grounds, there is, in fact, bound to be a gradient of activity in such a tubular system as the primary "coelomoduct". This tube was initially concerned with modifying the coelomic fluid passing along its length so as to prevent loss, from the animal, of salts and useful metabolites, while yet allowing excretory products to escape and maintaining a free passage for the germ cells. As the fluid flowed down this tube, its composition was modified and the activities of the cells of the walls must have been modified accordingly. The gradients of alkaline phosphatase activity, of esterase activity, of siderophilia, of fuchsinophilia, of sudanophilia, of mineralo- or gluco-corticoid production and so on, as they are seen in the kidney tubules or in the cells of the adrenal cortex, may not only be related directly to the tubular contents, as they probably still are in the kidney, or to the composition of the blood flowing in the vessels parallel to the columns of cells as they clearly do in the adrenal cortex, but they may also owe their presence to the gradients in the tubular contents in the ancestral forms and may now be maintained as an inherited characteristic. The gradient of activity could follow any of the lines suggested in the diagram (Fig. 16.3), and the results, with respect to the adrenal cortex, would give the corresponding histological pictures. In other words, the adrenal cortex of mammals represents a very

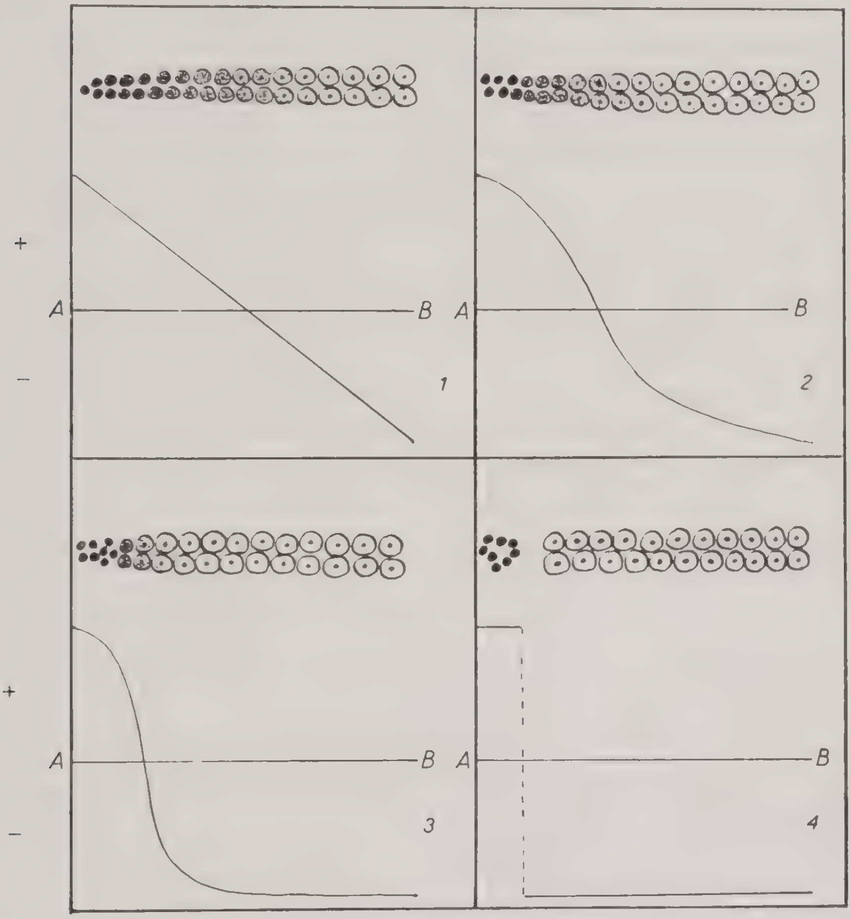


FIG. 16.3.

The effect of alterations in the "gradient" on the morphology of the adrenal cortex. The line AB in each case represents the change-over from + (i.e. "water elimination-cation conserving") activity to — (i.e. "water absorbing-cation ejecting") activity.

⊙ "Intermediate" cell. ● Typical glomerulosa cell. ⊙ Typical fasciculata cell.

similar arrangement of cells to that from which it was probably derived, namely, a series of tubes or columns of cells, each column showing a gradient of activity with respect to salt balance and conservation of essential metabolites. Since the tubular character of these cell systems has largely disappeared, the salt balance itself must now be an indirect activity. Nevertheless, changes in the salt environment of these cells on the side towards the capillaries, i.e. the basal pole of the cell, but more particularly on the other side, could cause changes in their metabolic processes and these could then lead to the production or liberation of metabolites which have acquired importance as hormones (Deane, Shaw and Greep, 1948; Vogt, 1951). Instead, therefore, of being directly concerned with salt and metabolite equilibration as they probably were at one time, the metabolism of the adrenal cords now becomes modified by the local conditions in which their cells are placed, and the by-products of the cells can then become available as hormones to modify the activity of other tissues. In this case, the particular by-products are presumably the mineralo-corticoids (e.g. aldosterone), steroids secreted mostly by the zona glomerulosa cells in relation to the Na^+/K^+ ratio of the blood (Stoerk, Knowlton and Loeb, 1955; Rosenfield, Rosenberg, Ungar and Dorfman, 1956; Rosnagle and Farrell, 1956) or to the total volume of the blood, and the glucocorticoids, steroids mostly secreted by the zona fasciculata in response to stimulation by the secretions of the pituitary which are concerned in regulating the glucose content of the blood. Translated into other terms, if the glomerulosa hormones tend to favour the production of salt-conserving, water-eliminating activity (cf. flagellate, ciliate cells) then the fasciculata cells might favour the glucose-absorbing mechanism (cf. brush-border cells). Perhaps the electron-microscope observation (Carr, 1958), that microvilli exist in numbers on the surface of adrenal cortical cells of the zona fasciculata at those points where two or three cells meet at an angle, could be interpreted to mean that this space is the remnant of the initial lumen of the tube and that the microvilli are all that is now left of the original brush border. It might also be suggested that reticulata or X-zone cells, if they represent the cells at the other extreme of the gradient, could logically be expected to produce substances which encourage salt ejection and water-inbibition, i.e. to act in opposition to the hormones of the zona glomerulosa; but other machinery may exist for that purpose (see p. 347).

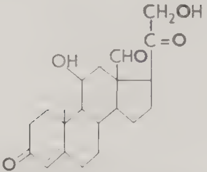
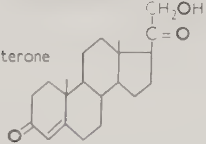
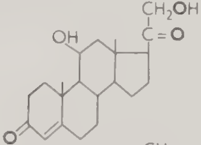
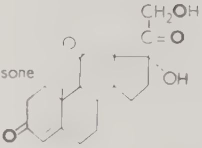
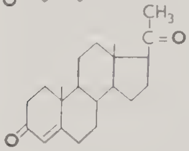
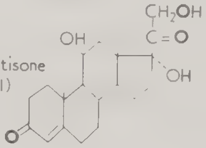
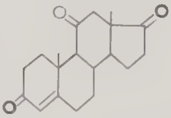
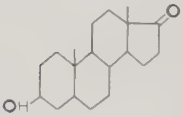
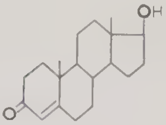
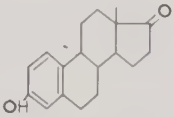
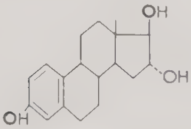
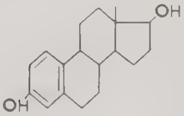
There is one aspect of the gradient system which may perhaps be emphasized in relation to the possible part which steroids play in the economy of the cell. The blood flows through the adrenal cortex from the capsule towards the medulla. Presumably, therefore, the oxygen tension falls progressively towards the medulla, i.e. along the length of the tubes. This may or may not be connected with the character of the steroid produced, but certainly if the steroid molecule is thought of as a long molecule whose end groups are the important groups, then they can be arranged in a series with the more highly oxidized members near the capsule and the less oxidized members nearer the medulla. In other words, there is a gradient in the degree of oxidation of the end groups of the steroids from a high state of oxidation near the capsule to a much lower state near the medulla (see Table 16.1). The greater quantities of ascorbic acid in the deeper layers of the cortex is perhaps another indication of the same thing.

STEROID METABOLISM

The occurrence of steroids in all these tissues is certainly something more than an interesting phenomenon. Apart from the liver, which is perhaps to be regarded as a tissue placed in a very special metabolic position in the body, the occurrence of steroids in any quantity is largely confined to the cells which are now under consideration as coelomoduct derivatives. It is as if these cells had at some very early stage developed some twist of their metabolism, which either led to, or required, the production or accumulation of the steroid molecule in some form or another. Once this trait had developed, it persisted and has since been modified in a variety of ways. In some derivatives of the original cells, it may be strongly developed; in others, it may only be latent, as the following examples show.

When cholesterol is injected into the peritoneal cavity of the salamander, it is found that it is picked up, probably from the lumen of the nephron, by a particular part of the proximal tubule of the kidney (Gérard and Cordier, 1933). In other respects normal kidney tissue does not generally show much in the way of steroids in the cytoplasm of its cells. Exceptionally, however, as in the kidneys of the Carnivora, and of the Felidae in particular, the cells of the tubules, especially of the proximal tubules, may be heavily laden with fat particles (Hewer, Matthews and Malkin, 1948) which give positive tests for steroids (Lobban, 1955). In the dog a similar situation is found (MacNider, 1945), but in this case the location of the fat is in the distal part of the proximal convoluted tube, while in the cat it is in the first segment of the proximal tube after the neck-segment (Foote and Grafflin, 1942). It is true that the finding of steroids in the kidneys of the Carnivora is an exception and may only reflect some peculiarity of steriod metabolism elsewhere in the body or some peculiarity in the

TABLE 16.1

Zona glomerulosa	Aldosterone			
	Desoxycorticosterone			
Zona fasciculata	Corticosterone		Cortisone	
(Corpus luteum)	Progesterone		Hydrocortisone (Cortisol)	
	Adrenosterone			
Zona reticulata and X-zone (Testis)	Androsterone			
	Testosterone			
(Ovary)	Oestrone			
	Oestriol			
	Oestradiol			

method of excretion of these substances. The avidity of these cells for steroids is nevertheless very real and these exceptional cases may be very significant in relation to theories of evolution; a potentiality may be latent in a tissue and yet only rarely become manifest as in this case, or in other cases, it may be nearly always realized and only exceptionally remain latent. An example of the latter is found in the golden hamster. In most animals, the cells of the adrenal cortex show large quantities of visible fatty material in their cytoplasm, and in this fat, cholesterol and steroids can generally be detected, but in the golden hamster, for instance, the adrenal cortex normally shows no histological signs of the presence of steroids or of cholesterol (Alpert, 1950).

Within the cells of the ovarian follicle, steroids, though present, are not there in large amounts, but immediately after ovulation the cells start to store large quantities of lipids. The follicular fluid is, of course, a rich source of oestrogens. There are several factors involved here, e.g. the formation, segregation, liberation, and storage of steroids, their precursors and their breakdown products. Certainly, in the adrenal gland neither the visible content of the cells nor the amount of active material which can be extracted from the gland is any indication of the amount of hormone which the gland is producing and ejecting into the blood stream. The production of these hormones may thus be analogous to that of the mucopolysaccharides as discussed in earlier chapters. Many cells may be able to form them, but only some liberate them externally to themselves, while others accumulate them within the cytoplasm and perhaps liberate them in bulk only at intervals. The presence or absence of steroid material in histological sections is not therefore by itself necessarily a true indication of the actual functional activity of the tissue at the time. Nevertheless the frequent presence of steroids in the cytoplasm is certainly an index of the potentialities of the cells, and the presence of steroids in easily detectable amounts is a very characteristic feature of many of the tissues under discussion. In this connexion, attention may profitably be called to some recent work on the structure of mitochondria, for again the steroid-storing cells have their peculiarities. The electron-microscope shows that most mitochondria are surrounded by a membrane, which incidentally is generally double, and within the membrane there are developed numerous "cristae" which project towards the centre of the mitochondrion like shelves on the wall of a cupboard. In steroid-producing cells (fasciculata and reticulata cells of adrenal cortex, luteal cells of ovary, interstitial cells of testis) these cristae, instead of being flattened, are tubular in section (Belt and Pease, 1956).

It is too early to conjecture how significant this is. Other cases where a similar structure is found are in the mitochondria of certain ciliates (*Euplotes* and *Paramecium*), in the oviduct of certain snails (*Helix*), and, of special interest here, in the cells of the Malpighian tubes of the grasshopper (see p. 274) and, mixed with cristae, in the kidney of the rat (Beams, Tahmisian and Devine, 1955; Powers, Ehret, Roth and Minick, 1956). It is possible that the tubular form represents the more primitive condition and that cristae are a secondary development or that the form of the mitochondrion is related to the same phenomena as have culminated in the production of mechanoblasts and amoeboblasts. This is suggested by the observations that *Amoeba proteus* has very well developed microvilli, *Tetrahymena* has smaller microvilli and some cristae, while *Euglena* and *Poteriochromonas* have cristae (Sedar and Rudzinska, 1956). The ciliates are, however, a stumbling block on this pathway.

The upshot from all these considerations therefore, is that the kidney cells, the adrenal cortical cells, and the supporting or interstitial cells of the gonads constitute a family of cells based on their origin from "coelomoducts". Their behaviour is determined partly by their origin and partly by their environment and the work they are called upon to do. They have certain common biochemical and cytological peculiarities, such as the tendency to accumulate steroids and, to a lesser extent, carotenoids, and have certain common morphological features, such as the tendency to form vesicles, tubes or cords of cells; and these tubes display definite gradients of activity. Indeed, whether the structure is that of a vesicle, tube or cord may depend, at least in part, upon the direction of activity of the cells in relation to the transfer of fluid across the cell. The cells respond readily to changes in the composition of the fluids bathing them; they are essentially regulators of their environment.

The situation in the Canadian porcupine, which was investigated by Mossman and Judas (1949), is particularly illuminating in this respect. In the ovary of this animal there is an unusually large number of interstitial cells which, as in other species, just before oestrus, develop into well-marked thecal glands. At this stage, there is a clear distinction between granulosa cells and thecal cells, and the two are separated by connective tissue; the position is at least superficially somewhat comparable with that in the bovine adrenal cortex; in both cases there is a sharp distinction between the vesicular part of the "tube" or "cord" and the rest. At ovulation in the porcupine, this state of affairs suddenly changes; both cortical (i.e. follicular cells) and medullary (i.e. thecal) cells immediately enlarge and become luteal cells without much visible distinction between them, and both start to store lipids. They behave as though they had changed their place in the "gradient"—presumably the factors causing the initial gradient have in fact changed, by the process of ovulation.

INTERACTION BETWEEN "COELOMODUCTS" AND THE CONTROL OF THEIR ACTIVITY

As in any family there is some degree of integration, or at least of interaction, so it is likely that the activities of the ancient coelomoducts were always more or less integrated with, and sooner or later related to, those of nephridia. Hyperactivity in one part would necessarily have repercussions on the activity in another part particularly since, as seems likely in such a polarized or directional system as these tubes must have constituted, the activity must involve localized changes in tissue-fluid composition. If, to take a simple example, a tube in one part of the animal absorbs Na^+ very strongly and pours it into the blood, then any cell bathed by that blood will have to do even more work to increase the Na^+ content of the blood still further, and in this sort of way the activity of cells in a single coelomoduct or the activity of a single unit in a series of coelomoducts, may be to some extent self-regulatory. As mentioned earlier, not only their direct actions but also the by-products of their activities may also be used as co-ordinators, either in addition to, or instead of, the object of their direct action. For example, there is good evidence that secreted corticoids inhibit their own further production by cells of the adrenal cortex *in vitro* (Birmingham and Kurlents, 1958). This problem of integration calls for further consideration and for an examination of the origin and function of tissues further afield than the immediate urinogenital complex and will form the subject of the next chapter. There is, however, another possible reason for the sort of integration that is found, for example, between the adrenal cortex and the kidney. From the point of view of the body as a whole, the important property of the kidney, with respect to the chief blood constituents, is that it should maintain their concentration at a constant level, and this in spite of the fact that their intake may be extremely variable. The problem may be illustrated by considering the Na^+/K^+ ratio in the blood and the excretion of sodium. According to the intake, sodium may or may not appear in the urine. This means that the cells of the distal tubules of the kidney, which are those probably concerned with the fine adjustment of the sodium-potassium balance, may have their tubular surfaces bathed with high or low concentrations of sodium as the case may be. The pole of the cells towards the tissue fluid is required to adjust the activity of the cell so that the tissue fluid is given exactly the right sodium-potassium ratio. This might be possible for one cell, but it would be difficult, one imagines, to organize the activity of all the kidney cells to respond in such a "dead-beat" or absolute fashion to the composition of the blood in the renal vessels, so that they all added the sodium to the fluid to just the right amount and no more. In addition to this they would never be able to repair a serious Na^+ shortage quickly, since they would never be able to raise the local blood-sodium level above normal, even though the blood coming to the kidney was grossly deficient. On the other hand, if those cells of the adrenal cortex which originally were involved in this same regulative activity altered the amount of their hormone production according to the blood Na^+/K^+ ratio, and if this hormone circulated to the kidney and controlled the reabsorptive activity of the kidney cells, then the system would be directly related to the blood and not to local Na^+/K^+ conditions within the kidney itself. One could visualize the system developing if, for example, aldosterone and deoxycorticosterone were substances which were necessary for sodium transport into a cell but which were eliminated from the cell during the transport or *vice versa*. Thus an adrenal cell, faced with a serious sodium shortage outside has to work to maintain its sodium content. In so doing, it liberates aldosterone or deoxycorticosterone into the blood and goes on doing so until equilibrium is established. The aldosterone then circulates to the kidney and assists Na^+ transport out of the cell into the blood by itself entering the cell where it could be inactivated, stored or excreted. This is probably altogether too simple a picture of the situation in which, in any case, the mineralocorticoids appear to be more involved in regulating the Na^+/K^+ ratio than the Na^+ alone. There is also good evidence that their action in this respect is not confined only to the kidney but involves other tissues also, as for example the parotid salivary gland, in sheep and cattle at least (Denton, 1958); nevertheless, it may serve to illustrate the possible advantages of a controlled excretion and the sort of way in which such a control system might perhaps have evolved.

There is some evidence that the zona glomerulosa cells, in the conscious and "unstressed" sheep at least, are not adequately stimulated to produce aldosterone directly by changes in the Na/K ratio in the blood bathing them, but that they, too, may depend on some as yet unspecified hormonal influence (Denton, 1958). When the secretory activity of the parotid gland is investigated, the ratio of Na^+ to K^+ in the secreted fluid can be used as an index of aldosterone secretion, and there is a lag period of about an hour before changes in the Na/K balance of the animal exert their effects, and direct perfusion of the adrenal gland with blood with abnormal Na/K ratios has unexpectedly small and delayed results. There is, however, one point to be considered in relation to the orientation of the cells of the zona glomerulosa; if they were originally concerned with the coelomic contents, then the Na/K ratio on their "coelomic" surface and not their vascular surface would presumably be the effective stimulus. This presumably means that the fluid in the vesicles of the zona glomerulosa would be the effective stimulus, if the sheep's adrenal is built on the same plan as that of cattle. Alternatively, it may be that some other structure, perhaps originally of coelomic origin also, now acts as a go-between. It may even be that the volume of the blood is an important factor.

Similar considerations of interplay between different parts of the "coelomoduct" complex could be made to apply, *mutatis mutandis*, for control of glucose and probably that of phosphate, calcium and the other constituents of the blood that are normally kept constant, and some suggestions along these lines will be made in the following chapter.

From the purely evolutionary aspect, it has been emphasized throughout all this discussion that there are two systems, coelomoducts and nephridia, whose functions, though initially very different, have become closely similar, and whose activities therefore must be integrated and co-ordinated in the interests of the organism as a whole. In investigating this integrated system, the idea that cell behaviour is determined by initial origin, by evolutionary history, and by present local conditions must provide the thread wherewith to extricate ourselves from the labyrinth.

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CHAPTER 17

COELOMODUCTS, NEPHRIDIA, KIDNEYS, GONADS, ADRENAL CORTEX AND OTHER DERIVATIVES

IN the last few chapters, certain relationships between the tissues derived from the genital ridge and the nephric rudiments have been discussed as consequences of their earlier coelomoduct or nephridial ancestry. It will be the purpose of this chapter to examine the relationships between these two tissues on an even wider scale and to study some of the implications they may have, with reference not only to the physiological functions of the tissues themselves, but also to the behaviour of certain other tissues which may have had a similar phylogenetic origin or derivation, and which may affect the mechanism of co-ordination between the urinogenital and adrenal functions.

The basic common pattern of all these tissues has been considered in terms of their probable derivation from one or other of the segmentally or serially arranged coelomoduct or nephridial systems. It has been shown that such tubes, whatever their origin, must ultimately have acquired certain properties and functions in common, though the mechanism by which they each acquired and originally performed these functions may have been somewhat different. Such differences, if they still exist and have not been modified beyond recognition, may be very important tools in helping to unravel the tangle of their origin, just as an investigation which would establish their origin beyond doubt would thereby help to elucidate the idiosyncrasies of their special functions.

The problem is a difficult one and its solution involves an ability to distinguish cart from horse under extremely nebulous conditions. Cells may acquire certain characters either because they are forced by their environment to function in a particular way, or because they happen to inherit the necessary characters and so be selectively used to fill a particular niche in the economy of the animal as a whole. In other fields, cases of convergent evolution have sometimes proceeded to extraordinary lengths to satisfy the demands of a special environmental niche, e.g. the swifts and the swallows, the marsupial dingo and the mammalian dog, the fruit eating hornbills of the Old World and the American toucans, or the snake-eating Cariamas of the South American prairies and the secretary birds of the South African veldt. We must therefore be prepared in cytology for corresponding booby-traps.

In the group of tissues under discussion, the actual form of any particular cell, e.g. a cell of the proximal convoluted tubule of the kidney, may have been achieved because the cell is performing specific functions, e.g. reabsorbing NaCl and glucose, or because it has inherited a pattern which especially fits it for doing these things. A combination of both is likely, i.e. a cell with certain capabilities has been selected and the capabilities have been further developed. As illustrated earlier, some of the cells of the Malpighian tube of a grasshopper have an ultrastructure, as revealed by the electron-microscope, almost precisely similar to that seen in the proximal tubule cell of a mammal, with brush-border processes, with very similar involutions of the basal surface and with tubular cristae in the mitochondria (Beams, Tahmisian and Devine, 1955). On the face of it this would look like a case of convergent evolution in that this is the form which all cells preferentially assume when handling NaCl and glucose absorption; in support of this the cells of the small intestine, where much the same activities are involved, certainly possess some of the same features. It might, on the other hand, be the inheritance of basic qualities that has caused these cells to be selected for the purpose; in which case, the cells of the Malpighian tube would, if traced back in their evolutionary origin, be found to be derived from the same stock as that which has elsewhere culminated in the proximal tubules of the kidney.

The special steroid metabolism connected with certain cells of the ovary, testis, adrenal cortex, and with the kidney of the carnivores, looks like the inheritance of a common peculiarity which has been modified towards somewhat different ends in each case, but it could also result from some environmental factor affecting the cells in that region of the animal or embryo. At the present stage it is important to bear in mind both possibilities, but it will be the purpose of the ensuing discussion to emphasize the consequences of the hereditary and evolutionary aspect, because, however much the environment may be able in the long run to bring about evolutionary changes, it nevertheless has to act on a pre-existing system, and the course of evolution is always likely to be determined by the nature and capabilities of the pre-existing system. All mammals have the same sort of calcareous skeleton, which was probably evolved in the first instance by the use of unwanted calcium salts to strengthen a pre-existing collagenous or cartilaginous framework; no mammal, nor any animal for that matter, has ever developed a skeleton with aluminium as the essential element. Aluminium is light, relatively non-toxic, and it would have many desirable features as a structural support, but it simply has not been used. It might perhaps be argued that it is difficult to extract aluminium from the food, but if certain tunicates can collect enough vanadium from the sea-water to use it in the formation of their blood pigment, it would appear to be not beyond the range of possibility for an animal to be able to construct a skeleton of aluminium or of several other possible inorganic substances for that matter; but skeletons have never evolved that way. Other suitable raw materials were more readily available, and the foundations for a calcareous skeleton were already laid. The case is exactly parallel with the evolution of the motor car from the Victorian coach and not directly from the Roman chariot or as an invention *de novo*. Tunicates are exceptional; and calcium is biologically an important substance, while aluminium is not. Moreover, examples of convergent evolution are the exception rather than the rule, and Nature proceeds more often by modification than by invention.

Thus, to return to the kidney-tubule cells, the question to be answered is whether any cell of an organism could acquire those particular characteristics or whether it is the prerogative of a certain group or family of cells to do so. From the study of *Naegleria* and of the sponge embryos, it seems to be a tenable hypothesis that, at an early stage in the development of an organism, the cells can be broadly divided into those which in their ordinary environment have need to conserve ions and those which can squander them. This probably means the same as a division into those whose ionic content is habitually below that of the environment and those in which it is normally above it. In addition to these main groups, there is likely to be an intermediate group, not so strongly biassed in one direction or the other, whose cells are capable of being changed by the environment either into one form or into the other, or of acquiring special significance for certain functions by virtue of the fact that part of the cell can be put into one state while another part of the cell is in the other: this is, of course, the condition in which a great many glandular or excretory epithelial cells must be placed; the distal tubule cell for instance may be absorbing NaCl on one surface and ejecting it at the opposite end. This situation could most easily arise among those cells which were "intermediate" with respect to their ionic equilibrium and which were so placed as to be presented with a different ionic environment at their two ends, and it would only arise with more difficulty in those cells that were more strongly biassed at an early stage in one direction or another. In all epithelia, necessarily separating two environments, the polarity of the cell is of the utmost importance. Furthermore, one end of the cell must presumably be dominant and call the tune to which the cell must dance.

This grouping of cells on the basis of their relationship with their ionic environment is obviously not in itself very helpful in relation to epithelia unless the polarity of the cells is known, but it may yet be very significant for grouping cells in the more stable environment of the inner mass of tissues, i.e. the cells of the true mesenchyme or mesohyl. It would seem probable that most epithelia could develop the necessary equipment of their surface to deal with altered salt environments and there would be definite advantages to the organism in having this reactive condition. A study, in relation to their ionic environment, of cells showing a "brush-border" or microvilli on one face and infolded plasma membranes on the opposite face, or of cells showing one or other of these, is certainly of interest, and some of the information so far available on this point has been set out in Fig. 12.5.

In the more stable and uniform environment of the inner cells of the body, the macrophage is characterized by the frequent occurrence of infoldings of its membrane (Palade, 1955), while the fibroblast is characterized by thin, pointed processes and filamentous pseudopodia. These two groups of cells may then, as already suggested, be considered as ion-ejectors and ion-conservers respectively in relation to their surrounding fluids. The epithelial cells from which they were initially derived are likely, therefore, to have belonged to the same categories with respect to the fluids of the body, i.e. the surfaces of the cells which were next to the "blastocoele" fluid must have been acting respectively as ion-ejectors or ion-conservers. Consequently, a study of the basal ends of epithelial cells in other situations may lead to a grouping of these cells from this point of view and is likely to be much more revealing and fundamental than the study of the apical ends, for the properties of these must be determined, at least partly, by the nature of the fluid with which the cells are in contact, and this is often likely to differ widely from that of the tissue fluid or blood. Fig. 12.5 certainly shows that many epithelial cells have a relatively smooth surface at the basal end while others have numerous infoldings. Whether anything like a brush border (i.e. with projecting finger-like processes) ever develops at this end is uncertain; at least it has not been described as such. This, however, may not be the character for which to look.

Mechanocytes tend to eject mucoproteins or some related substances (i.e. polysaccharides or their precursors) into their surroundings and together with these they tend to liberate the precursors of collagen. It may therefore be found that the epithelial cell of the premechanocyte type or mechanoblast, the ion-conserving epithelial cell, tends to produce more in the way of a basement membrane than the cells of the pre-amoebocyte, or ion-ejecting epithelial cells, and the study of basement membranes could then be revealing. Since the point has frequently been made that many epithelia have cells of more than one type, however, the basement membrane may reveal only the average capabilities of the epithelial cells and the local mechanocytes.

The Fate of Nephridia and Coelomoducts other than those Incorporated into the Urino-Genital System

However this may be, it is likely that the cells which originally entered into the formation of nephridia and coelomoducts developed their regulatory capacities (using the term here in its widest sense even to include such processes as glucose-absorption as well as absorption or excretion of ions) to the full. This being so, it is necessary to examine the cells in tissues which may have been derived from these same sources to try to decide whether they are primarily absorbers or excretors with respect to the tissue fluids, because the two are likely to have very different characteristics. This problem has to be faced whether the tissues under discussion are derived from nephridia, from coelomoducts or from some combination of both.

In addition to this basic quality of directional ion movement dictated by the ionic concentration of the tissue fluid, which must impose certain characters on the cells, the nephridial cells, as already discussed, may well differ in other metabolic traits from the coelomoduct cells. However, in the simplest of terms and in relation to their primary functions of osmoregulation and conservation of useful diffusible metabolites (e.g. glucose), nephridial cells could be expected to have become specialized for (a) water excretion and/or sodium retention, (b) water retention and/or sodium excretion, and (c) glucose absorption. The cells of coelomoducts may well have been selected on similar grounds and their activities fall into the same three groups. Therefore, if both systems of tubules have survived and have been incorporated into the urinogenital system of the vertebrates, there may be at least six main types of cell represented in the kidneys and other organs under discussion.

The kidney is, of course, the primary organ for the regulation of the blood by selective excretion, but it has been shown that the adrenal cortex and interstitial cells of the sex glands are of the same stock and, while no longer concerned directly with excretion, they are of great importance indirectly in regulating the composition of the blood. All these organs are embryologically derivatives of the easily recognizable "coelomoduct" system but, curiously enough, they only arise from a part of it and develop in only a comparatively few segments of the body. It is obviously of the greatest importance, therefore, to examine the other segments of the body for traces of nephridia, true coelomoducts or nephromixia. This search must be broadly based and must not only involve embryological investigations of peculiarities of development, of vestigial organs and the like, but also biochemical and cytochemical investigations of peculiar characters of metabolism and of cell polarity. It must seek direct evidence of similarities of function, and must analyse any feed-back mechanisms and mutual interrelationships and antagonisms which may be encountered between apparently unrelated cells in different situations.

For example, the gill arches in vertebrate embryos are more or less segmentally-arranged structures, and associated with them there develop structures of doubtful origin and significance: epithelioid bodies, postbranchial bodies, thymus, parathyroids, "chloride-secreting cells", carotid and aortic bodies, and even the thyroid and certain aggregates of "brown fat" should perhaps be included in this "pharyngeal complex". The functions of some of these derivatives are only rather vaguely ascertained as yet, but at least the parathyroid, the chloride-secreting cells of fishes and the carotid and aortic bodies are directly concerned with the regulation of the composition of the blood. It is therefore a matter of some significance that *Amphioxus* has actual nephridia on its gill arches and these are not represented as such in any of the vertebrates; this is certainly curious if the gill arches of *Amphioxus* are homologous with those of vertebrates. The possibility of there being developmental relationships between these miscellaneous organs and obsolete nephridia is therefore very relevant and will merit further discussion.

At this stage in our knowledge it is not possible to do much more than make suggestions as to the sort of information which is required for the solution of this problem. It is not a new idea that the tissues mentioned, and indeed others besides, have all arisen by modification of a segmental system of "excretory" tubes. The idea was first developed by Gaskell in 1903 in one of his series of papers on the "Origin of Vertebrates". He pointed out the similarities between the segmental urinogenital ducts of the primitive vertebrates and the coxal glands of *Peripatus*, and put forward the hypothesis that the vertebrates had developed from some segmental organism which basically had excretory ducts (coxal glands), belonging to each appendage. He also pointed out that the strictly segmental arrangement might have been partly obscured by the fusion of segments or the loss of appendages, or by their modification, such as has occurred, for example, in the arthropods. Nevertheless, he considered it likely that some remnants of the coxal glands themselves would often remain, although in some secondary or modified form. From these modifications Gaskell suggested that most, if not all, of the ductless glands had their origin. "By this simple explanation", he said, "we see how all these hitherto mysterious ductless glands, pituitary, thymus, tonsils, thyroid, are all accounted for, are all members of a common stock—coxal glands—which originally, as in *Peripatus*, excreted at the base of the prosomatic and mesosomatic appendages."

In the light of later work, while many of us would nowadays find it difficult to agree with Gaskell in some of his hypotheses, on this particular point there can be very little doubt that he was thinking along the right lines, though again probably incorrectly in detail. Vertebrates must have arisen from some stock with marked segmentation, or at least serialization, although this may have been already partly obscured by specialization in the anterior region of the body, and it is extremely likely that each segment originally had its own excretory and/or genital system involving a coelomic cavity and coelomoduct. These urinary and genital tubes may have been condensed, combined, or in other ways modified, as has certainly happened in the annelids, which are animals probably deriving from a somewhat similar ancestor to that which gave rise to the vertebrates. In general, the tubes are unlikely to have been entirely lost; embryonic rudiments at least may be expected which might or might not be followed by later modifications in some new direction.

The Nemertine as a Prototype

This is not the place in which to discuss the origin of vertebrates, nor to try to assess the claims of the various theories that have been put forward, but at the same time attention may be called, as it was by Hubrecht in 1883, to some of the ways in which the nemertines at least foreshadow certain very characteristic vertebrate or chordate features. Hubrecht himself called attention to the ectodermal origin of the nervous tissue and to the existence of commissures dorsal to the alimentary canal, and even suggested how the vertebrate nervous system could develop by approximation of the lateral nerve cords. Reference has already been made to the relationship between the excretory organs (nephridia) and the vascular system and to the linking together of the nephridial ducts into a common excretory duct. The presence of haemoglobin in the blood is not diagnostic of relationship to the vertebrates but nevertheless is interesting. The simple segmentation or repetitive pattern shown by the coelomic sacs with their gonoducts, is exactly the sort of segmentation that appears in the probably rather specialized and even degenerate *Balanoglossus*, an animal showing certain well-recognizable pre-chordate features. If the vertebrates were initially derived from some nemertine-like organism, it might be expected, since nemertines have both nephridia and gonoducts, that remnants of both systems would persist in the manner in which they actually seem to have done, e.g. nephridia in *Amphioxus*, and pronephric funnels (coelomostomes) in cyclostomes and higher forms. Finally, the early embryology of nemertines is unspecialized and is in many ways closely similar to that occurring in the echinoderms, animals which now seem to have fallen from grace as possible originators of the vertebrate stock, though offering striking similarities in their embryonic forms (Barraclough Fell, 1948). It would be interesting to know whether any of the nemertines ever show any creatine phosphate (*Lineus* contains arginine phosphate) which, in some quarters, is regarded as a biochemical step towards vertebrate specialization. Furthermore, a particularly interesting feature of nemertines is a curious ciliated groove or tube which develops from the ectoderm in close connexion with one of the main lobes of the brain. Rathke's pouch in vertebrates is also a ciliated pit that develops from the ectoderm and approaches the brain. The homology of the notochord with the nemertine proboscis has also been suggested.

It would thus appear to be a plausible hypothesis that organisms with certain of the features which are exhibited in nemertines, and at about the same level of organization as a generalized member of this group, could well have had features which were destined to radiate in a variety of specializations towards annelids, arthropods, echinoderms, or chordates. Potential segmentation or serialization could become the rigid metameric segmentation of the arthropods, lateral nerve trunks are capable of moving dorsally or ventrally and some nemertines already have dorsal nerve cords as a potential chordate character. Repetitive coelomoducts and repetitive nephridia could develop in endless variety.

If this hypothesis is correct, then these creatures are likely to be among those living at the present day and still showing something of the nature of the raw material from which "higher" forms have developed, and they would well repay closer study from this point of view. Moreover, it is evident that throughout the length of the vertebrate body some traces of the repetitive organs should be expected, though often in greatly modified form. Thus Gaskell was probably right in suggesting that the excretory organs (though not necessarily the coxal glands as such) would become modified, if no longer required directly for excretory functions, and would give rise to such things as the parathyroids, carotid bodies and thymus, and with this group he also included the lymphoid tissue in general. On functional grounds alone it would not seem at all unreasonable to believe that excretory or tissue-fluid-regulatory organs should subsequently become specialized as Ca^{++} or PO_4 controllers or as pH-sensitive receptors. The connexion with lymphoid tissue is not so immediately obvious, but again he may well have been right, as the following points will show.

Lymph Glands and Lymphocytes

In addition to the observations already made (see p. 263) on the behaviour of the pronephros of the larval lamprey, Maas (1897) has demonstrated that the myxinoid cyclostome has a curious development of the pronephros in that the pronephric funnels, which as usual lead out from the coelom, do not make connexion with the segmental duct but end, apparently blindly, in connexion with a network of blood vessels. Price (1910) and Conel (1917) have followed up this observation and, in the species which they examined (*Bdellostoma stouti*), have been able to show that the tubules eventually open into the cardinal vein. In these creatures, isolated glomeruli may also occur in the pronephric region, but, in general, the blood vessels ramify around the tubular part of the pronephros. This connexion between "coelomoducts" and blood vessels has already received comment (p. 250), and may even date back to organisms no more complicated than the nemertines. Here it is again, turning up in another form, and Price has suggested that its function is now very different. He considers that in these primitive cyclostomes the pronephros is undergoing the change which, in a sense, explains the origin and primary function of lymph glands and to some extent of the lymphatic system. The coelomic fluid in simpler organisms, may in part represent tissue fluid or it may be specially secreted; but, as the coelomic cavity enlarged in higher forms, the provision of its fluid offered more of a problem, and this requirement seems to have been met by exudation from glomerular blood vessels. Owing to the large size of the cavity and to its close proximity to most of the tissues of the body, by-products of tissue metabolism would also be likely to enter the coelomic fluid. In *Bdellostoma* it is suggested by Price that this fluid is returned to the cardinal vein by means of the ciliated funnel driving it through a network of tubes as a sort of filtration mechanism which has all the essentials of the lymphoid glands of higher forms.

In *Myxine*, this behaviour of the pronephric tubules is even more interesting, in that the lymphoid tissue, in the developing animal.

appears to undergo alternating phases of being purely lymphoid and phagocytic and of being actively haemopoietic, even to the extent of producing erythrocytes. The cells of the tubules themselves appear to be able to give rise to haemopoietic cells on the one hand and to more fibroblastic cells on the other and, at certain stages in development, practically the whole of the pronephric tubule apart from the coelomic funnel is changed into this haemopoietic tissue (Holmgren, 1950) (Fig. 17.1). A detailed cytochemical and physiological analysis of this curious phenomenon might be remarkably illuminating. The large cells in the pronephric tubes that give rise to the haemopoietic cells, remind one of other large cells in the urino-genital complex.

The connexion between nephric tissue and lymphoid tissue is also well supported by the fact that the "head-kidney" or pronephros of some teleosts, which develops in a very similar way, may in the end also become converted into a mass of lymphoid tissue (Rasquin, 1951) Sometimes an occasional tubule may be present as such, but the pronephros is mostly lymphoid tissue with some patches of adrenal tissue mixed up with it.

Moreover, there are certain observations on other animals which seem very relevant in connexion with the origin of lymphoid tissue. In *Phoronis* (Goodrich, 1903; 1945), when the larval nephridium, whose solenocytes project into the haemocoele, ceases to function as the organism matures, the solenocytes are shed into the blood as small basophilic, lymphocyte-like cells. In the pronephros of the ammocoete of *Lampetra* (Gérard, 1933) the tubules, as in the myxinoids, do not reach the outside world but open into what is apparently a blood-forming tissue with numerous athrocytic cells, and their contained fluids eventually drain into the cardinal veins. This tissue is, again, essentially lymphoid in character and probably haemopoietic. Furthermore, in the branchial region of the ammocoete, each arch has a group of cells that are also strongly phagocytic and have a pronounced capacity for taking up iron. Are these, one wonders, all examples of nephridia that have ceased to function as such, but whose cells have taken on the functions of lymphoid tissue? It has already been suggested that other cells from the same source continue to function in connexion with the regulation of the body fluids, as parathyroid cells, thymocytes, carotid-body cells, chloride-secreting cells, and the like, and this inclusion of lymphoid tissue in the same group could be interpreted to mean that lymphocytes are primarily concerned with some aspect of homoiostasis which had its origin in excretory nephridia. The possible connexion between lymphocytes and antibody formation and the mucoprotein nature of the "blood group substances" provoke interesting speculations in relation to this changed function of cells, which were originally concerned with ion transport and homoiostasis.

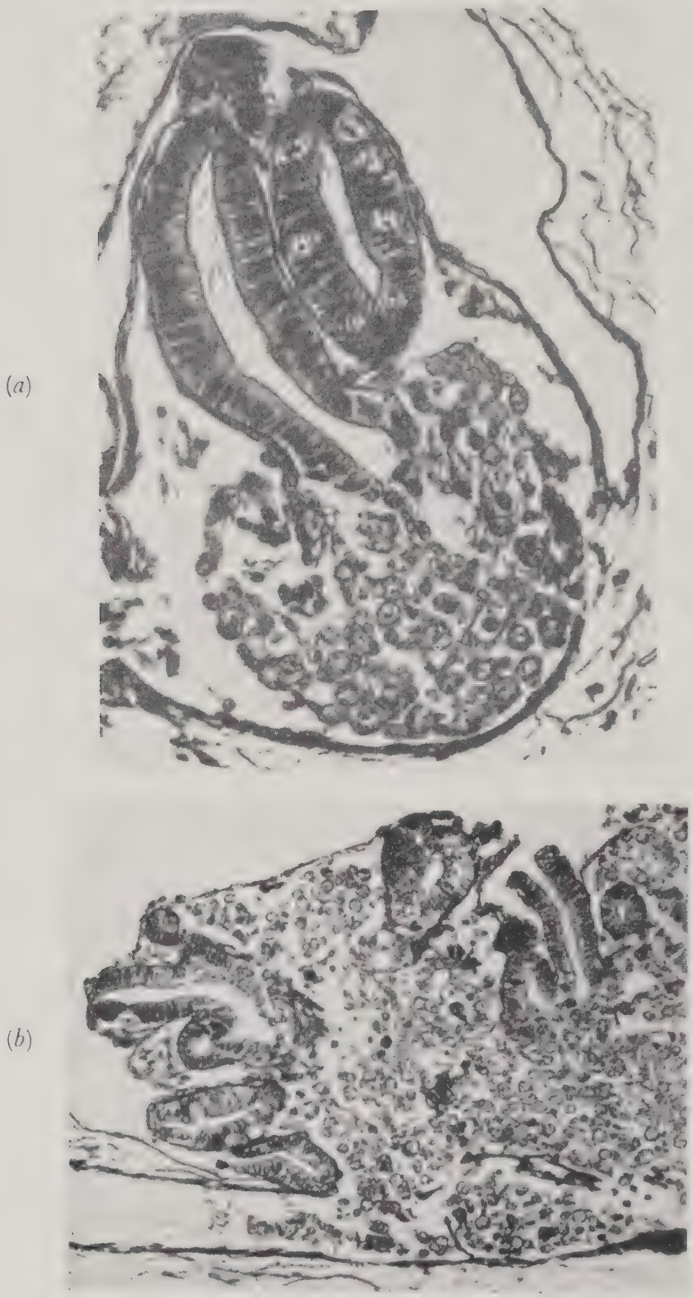

FIG. 17.1

Lymphoid and haemopoietic changes in the pronephric tubules of Myxine. (*a*) Pronephros of a 9.6 cm specimen. Haemocytoblasts and proerythroblasts in the central mass. Formative cells (haemocytoblasts) in the right tubule. (*b*) Pronephros of a 10 cm specimen. Central mass pouring its contents into the pronephric vein. (Holmgren, 1950).

Moreover, if the original solenocytes are the cells which have become the lymphocytes it is quite likely that they would have the capacity for ejecting muco-substances from their surface in the same way as mechanocytes possess it (see Table 11.1).

It is worth noticing also that inter-renal tissue in many fish is closely associated with the walls of the cardinal veins, but the nature of this connexion is somewhat different from that here described in connexion with glomeruli and these "lymphoid" tissues.

In the pronephros of *Bdellostoma* (Price, 1910) there are three distinct groups of cells, apart from those actually connected with the vascular system. There are flagellate cells lining the funnels; secondly, there are more cubical cells associated with that part of the tube which comes into contact with the blood capillaries and forms the quasi-lymphoid tissue; finally, the cells of the segmental duct, where this (or remnants of it) is present, are clearly recognizable as a separate group and, in some cases at least, possess a brush border on the side next to the lumen. A study of the cytochemistry of these cells would probably yield some important information. So, too, would a more detailed comparison between the adrenal-cortical rudiments in Myxinoidea and those in the Petromyzontia be profitable. Members of the former group have blood whose salt content is comparable to that of the sea-water in which they live, while the lampreys have some powers of osmotic regulation and a much more dilute blood.

It is significant that in the teleost *Astyanax*, the head-kidney, which is represented by a large mass of lymphocytes, is sensitive to the injection of adrenocorticotrophic hormone like the lymphoid tissue in higher forms. The lymphocytes disappear and large numbers of macrophage-like cells appear (Rasquin, 1951). At the same time, the so-called true inter-renal tissue hypertrophies. Presumably the action on the lymphocytes is secondary to that on the inter-renal tissue, as that on the lymphoid tissue in mammals is secondary to the action on the adrenal cortex. In mammals also, ACTH probably acting through corticoids, brings about an immediate reduction in the lymphocytes and an increase in the macrophage-like cells in the lymphoid tissue and in the thymus. The lymphoid tissue also shows two other changes of interest: it becomes edematous, and also the macrophage-like cells which are not very actively phagocytic develop a brownish pigmentation and giant cells with "foamy" cytoplasm appear (Dougherty and White, 1945). It may be relevant at this point to note that the phagocytic activity of macrophages has been recently shown to be increased by oestrogens, and depressed by cortisol (Bilbey and Nicol, 1958; Nicol and Bilbey, 1958).

In diabetes insipidus and in stimulation of the adrenal cortex by ACTH, the zona glomerulosa decreases before the increasing zona fasciculata, and many of its cells become small, rounded and somewhat lymphocyte-like. However, these situations are far too complex to analyse at the cellular level by experiments on the whole animal. Now that "organ-culture" techniques are available, adrenal cortical tissue should become more susceptible to direct analysis, and the influence of each of the numerous factors which could be involved in its activity more properly assessed. It is perhaps important to emphasize, however, the relationship between lymphocytes and the pronephros on the one hand, and lymphopenic agents (e.g. cortisone, etc.) and the adrenal cortex on the other. The cells which have entered into the formation of the lymphoid tissues of cyclostomes and fishes appear to be those which, under other conditions, form some part of the kidney or adrenal-like tissue, unless, of course, the lymphocytes have originated elsewhere and invaded these particular places. Lymphoid tissue does not seem to occur as extensively in the invertebrates as in the vertebrates. It occurs in connexion with the stomach of crustacea (Fischer-Piette, 1931) and in one or two other places where, again, it is fairly clearly a modification of nephridial tissue. This perhaps indicates that lymphocyte-production is always a faculty of the "coelomoduct" tissues and that it is of some particular consequence to the animals of the vertebrate stock. Phagocytic cells occur in the nephridia and coelomic funnels of some annelids, but these are probably different in kind. Whatever lymphocytes now do in the general welfare of the organism (for a discussion of this see Trowell, 1958) these activities probably had their origin in something which the cells, constituting the nephridia, coelomic funnels or their ducts, were doing when they were acting as regulatory organs. This selected activity was not necessarily the primary function of the cells in question but was more likely to have been some sideline.

A case could be made out both for the relationship between lymphoid tissue and coelomic funnels, and also for that between lymphoid tissue and solenocytes or associated nephridial cells. In the pronephric lymphoid masses, the lymphoid tissue could perhaps be considered as being, like the lymphoid tissue of the gill arches, associated with modified nephridial structures. The lymphoid changes occurring in kidneys could also sometimes be so considered, and an investigation of the origin of the lymphoid cells that occur between the tubules of the aglomerular kidney of such fish as *Opsanus tau* (Fig. 17.2) might prove to be instructive. The lymphoid tissue in the adrenal cortex, on the other hand, and also in some kidney tissues, would appear to be more closely related to coelomic funnels; this being so, the lymphocytic form may be something which is easily derivable from the strongly "flagellate" type of cell, i.e. the type of cell which lies at the top of the ion-moving or water-moving gradient, the non-phagocytic "solenocyte", or mechanoblast type of cell. The macrophagic, athrocytic, phagocytic, amoeboid type of cell in the lymphoid tissue would then be considered as arising from the opposite end of the ion-moving gradient and so also could the salt-ejecting chloride-secreting cells on the gills of fishes. On these lines the action of ACTH on such a tissue as the adrenal cortex would certainly become more intelligible. ACTH strongly stimulates the zona fasciculata but has no action on or depresses, the zona glomerulosa. The destructive action on lymphocytes would clearly seem to fit firmly into the picture. Cortisone, as typical of the secretions of the zona fasciculata, acts on the lymphocytes so that they produce fluid around themselves (c.f. solenocytes) and become destroyed, perhaps by excessive functional activity, while the macrophage elements are fostered and become large and pigmented. This idea would, of course, lead to the suggestion that lymphocytes are more nearly related to the mechanocyte type of cell than to the macrophage, and this is a somewhat revolutionary hypothesis; but certainly their manner of directional locomotion by constriction rings is more nearly that of the fibroblast than that of the monocyte or macrophage (de Bruyn, 1946; Harris, 1953; Lewis, 1931, 1933). On

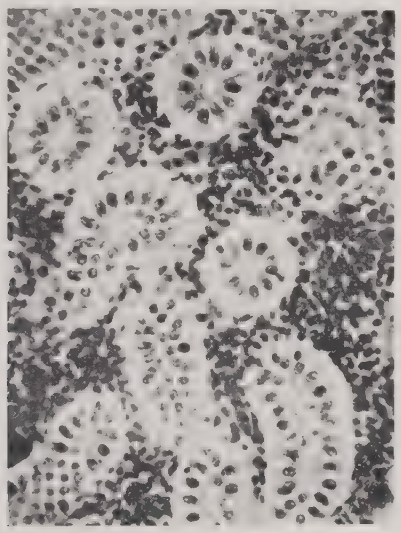

FIG. 17.2

"Lymphoid tissue" between the tubules of the aglomerular kidney of *Opsanus tau*. (Smith, 1951).

the other hand, the lymphocyte has periods of "rest" (Lewis, 1933) in which de Bruyn (1946) has shown that the cell is "depolarized" and is actually moving rather more in the manner of the macrophage. When the connexion between active transport of ions and the production of mucosubstances and collagen precursors as discussed in earlier chapters, is recalled, then the relationship between the adrenal cortical hormones and the diseases of the connective tissues becomes not only more intelligible but assumes the status of a logical consequence.

At this point the path becomes somewhat slippery because lymphoid tissue frequently develops in connexion with the salivary glands and the mucous and serous glands that open into the pharyngeal regions of the alimentary canal (Kingsbury, 1945). Does this mean that these glands also represent modifications of earlier nephridial structures? The idea is not impossible. There is the analogy with the earthworms, where Bahl (1947) has shown that nephridia do become modified into "salivary glands"; at least they open into the buccal cavity (see Fig. 13.14). The modifications of the salivary glands of mice in relation to sex (Junqueira, Fajer, Rabinovitch and Frankenthal, 1949; Raynaud and Rabeyrotte, 1949) and their sensitivity to aldosterone as in sheep, where the hormone decreases the ratio of Na/K in the secretion (Denton, 1958) perhaps become more readily comprehensible if these organs are to be considered as offshoots of the urinogenital complex.

These observations on the similarity of behaviour of nephridial and coelomoduct derivatives in relation to lymphocyte formation and so on, may, of course, have no more significance than that there are certain cells scattered all over the body, which are readily changed into lymphocytes (these could be the mechanocytes), and others (the amoebocytes) readily become specially active under the stimulus of the appropriate steroids.

If lymphocytes are connected with cells of the nephridial or coelomoduct group, then it is to be expected that they should occur elsewhere than in the strictly urino-genital system; in fact, potentially they could be present wherever cells differentiate towards the formation of "coelomoducts", and certain parts of the gill-arch tissue would, with their history of associated nephridia, obviously come into this category. Thymus, and perhaps tonsils too, would thus find some causal explanation for their particular situation, and the regression of the thymus associated with sexual maturity would fall into line with the similar and well-recognized modifications in the adrenal cortex and even in the kidneys as seen in fish, reptiles and Felidae, either in response to sex hormones as such, or in response to the gonadotrophic hormones originating in the pituitary.

THE HOMOIOSTATIC ENDOCRINE ORGANS

The close association of a capillary network with pronephric funnel tissue in fish, with glomerular tissue in kidneys, and with zona glomerulosa tissue in grafts (Williams, 1945), and even the maturing follicles of the ovary, indicates that the condition is not fortuitous and also provides a basis for one of the primary requirements of an endocrine gland. If this association is primitive, and certainly solenocytes often come into intimate association with blood vessels, then "coelomoduct" tissue is a very likely precursor for internally-secreting organs.

The parathyroid is a gland whose cells seem to have undergone a morphological change in some ways not unlike that which elsewhere has culminated in the formation of lymphocytes, but its cells are indubitably still endocrine, and endocrine in a very similar way to the cells of the zona glomerulosa of the adrenal cortex, i.e. they are concerned with ionic balance, but, in this case, with the control of the Ca^{++} and PO_4^{---} balance. This they may do in part by controlling the absorption of phosphate by the kidney tubules, which is certainly consistent with the view that activity in one part of the "coelomoduct" system would be likely to modify activity in another part, but tissue culture experiments by Gaillard (1956) have shown very clearly that at least part of their action is also directly related to bone tissue. Organ cultures of parathyroid tissue when placed in a suitable medium together with organ cultures of bone, induce the solution of the bone matrix and greater activity of osteoclasts. This sort of experiment, incidentally, shows the way, and possibly the only way, in which useful information can be obtained on the direct action, or otherwise, of hormones on target organs. In the endocrine system, as it is now becoming possible to envisage it, all the participants are so closely integrated that the action of any one is almost bound to cause secondary reactions in others, and only by limiting the system to the hormone producer and the suspected target organ is it possible to be certain of a direct action. Even then, a positive result is no criterion that the same action occurs in the body; for the environment of both hormone producer and target organ in tissue culture is not identical with the environment of either in the body. The action on the bone matrix is certainly interesting if the parathyroid cells are to be regarded as related to the cells at the mechanoblastic end of the gradient system.

On an earlier page the idea was put forward that the necessity for maintenance of the ionic equilibrium of a cell led to changes and adaptations in the surface layers of the cells. If ions were to be passed in one direction, this might only be possible if other substances, e.g. "muco-substances", amino-acids or sterols were also simultaneously and directionally moved. As a tentative hypothesis, cation-conservers were envisaged as ejecting glucosamine, or other amino-sugar or closely related substance, together with certain precursors of collagen, while cation-ejectors tended to store mucoproteins and other products within their cytoplasm. If this general idea is correct it means that cells can secrete without necessarily showing any evidence of storage of their secretion within themselves; thus, extracts of "glands" containing such cells might show only minimal traces of activity. By-products of the secretory activity might, however, accumulate in the cytoplasm during activity and become detectable as "secretion" granules or the like. The common assumption that granules in secretory cells always represent the secretion itself is therefore not necessarily valid; they may only represent by-products.

Of the systems under consideration, the zona glomerulosa of the adrenal gland, the parathyroids, the thymus and lymphoid tissue all have this feature in common that they have been considered as being comparable with the flagellate ion-conserver cells of the "coelomoducts". Such cells during activity would then be expected to throw off, into the surrounding tissue spaces, the by-products of their activity and these may subsequently have become their hormones. This being so, it would be more profitable to search the lymph from lymph glands and the blood from the thymus for active substances than to extract the glands themselves, just as the venous effluent from the adrenal has proved to be a more reliable source of cortical hormones than the gland itself. Antibody production by lymphocytes and plasma cells would seem to fit into this general picture as substances liberated by lymphocytes or plasma cells without evidence for their preliminary storage. The problem of secretion thus becomes connected directly with the nature of the cell surface and the stereochemistry of its constituents. This concept of secretion occurring on surfaces and of intra-cellular and extra-cellular secretion, is certainly supported on the one hand by the micro-structure of the cells of such organs as the pancreas, in which the nuclear surface or derivatives from it form the extremely elaborate endoplasmic reticulum with its accompanying "Palade granules" of ribosenucleoproteins, leading to the formation of intracellular secretions, and on the other by the liberation of HCl from the elaborate 'intracellular canals' of the oxyntic cells that are really portions of the outside world penetrating deeply into the cell (Fig. 17.3), or of antibodies by lymphocytes, or of "matrix" by mechanocytes. Perhaps a similar situation, emphasizing the directional nature of the cell surface with respect to secretion, is demonstrated in the location of an esterase in the different parts of the follicle of the ovary. With alpha-naphthyl butyrate as substrate, esterase is localized between the cells of the membrana granulosa, but within the cytoplasm of the cells of the theca interna and corpus luteum (Hunter and Kneiske, 1957).

The metabolism of carotid body cells evidently varies sufficiently in relation to the CO_2 content or pH of the blood bathing them to cause them to excite adjacent nerve fibres. On the basis of the hypothesis outlined earlier, this alteration of their metabolism presumably reflects some aspects of their original function when they formed part of a nephridium or coelomoduct. In the present form of these cells, as carotid body cells, the altered metabolism is turned towards another and accessory mechanism leading to the alteration of respiration and thus to the controlled elimination of carbonic acid, thereby relieving the remaining excretory or kidney tubules of some of their previous work.

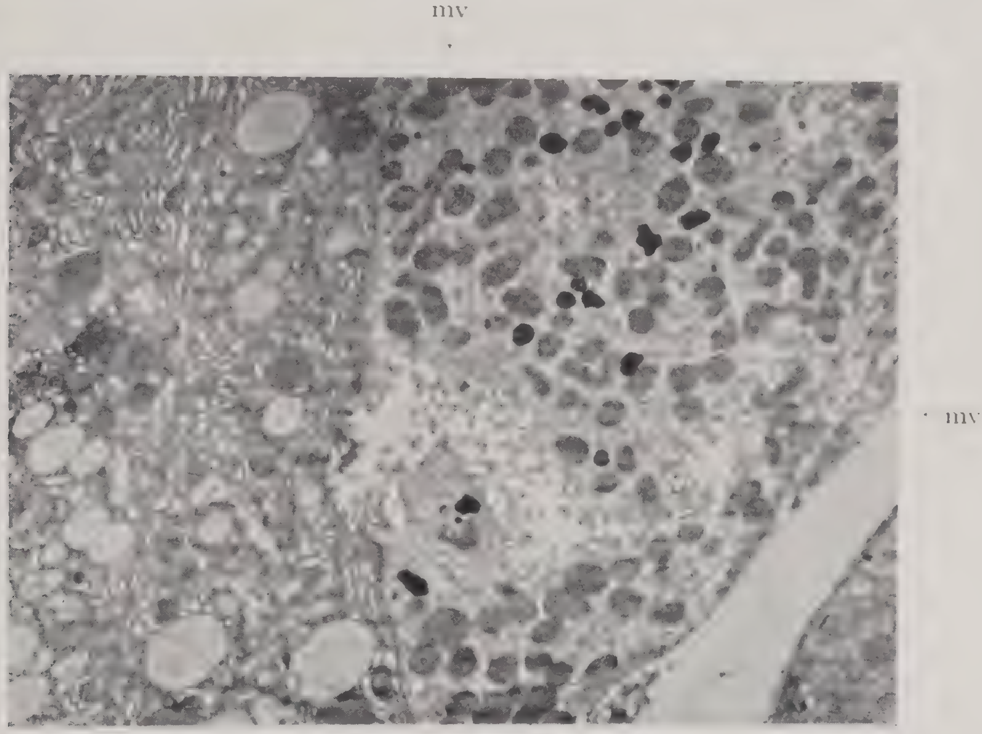

FIG. 17.3

Intracellular canals mv, with microvilli, in an oxyntic cell (right) of the stomach. The neighbouring secretory cells (left) show a very elaborate endoplasmic reticulum. The oxyntic cell has abundant mitochondria. (Photograph by courtesy of C. D. Hally, 1958).

There is clearly a *prima facie* case for considering at least one group of endocrine glands—which might be conveniently termed the homoistatic glands of the body—as being derived from the excretory and regulatory organs of their ancestors. The hormones of the alimentary canal can be conveniently regarded for the present as the products of a separate endocrine system, though their phylogenetic origin forms an intriguing problem. As homoiostasis became more complete, the different parts of the regulatory mechanism specialized for different aspects of the work: pH, Ca^{++}, Na^+, K^+, PO_4 , Cl , glucose etc., have each been taken over by special parts of the system. This specialization and consequent subdivision of the various parts of the homoiostatic mechanism would, however, have their dangers if the separate activities of the different parts were not in some way integrated. This integration is partly carried out by mutual inter-action and inhibition but the explanation for the peculiar and dominating position of the pituitary body as a "chief integrator" can now perhaps be seen to emerge.

The most anterior set of "coelomoduct" tubules may originally have acquired some control over the rest, simply by virtue of its anterior position in the body, in the sense that these tubules would be first to respond in any new environment into which the animal happened to be moving. If Rathke's pouch represents the remnants of the "coelomoduct" from the first coelomic cavity, as has been suggested (de Beer, 1926), then the anterior lobe of the pituitary has clearly evolved from this system. In the first instance then, it may have acted to some extent as a chemoreceptor organ of the external medium. Alterations in its action, leading to the liberation of by-products into the tissue fluids of the animal, could, if these by-products were utilized, provide the necessary stimuli for altered function of the more posteriorly situated "coelomoducts", so setting the general tone for the whole homoiostatic mechanism. Later, as the vascular system developed and as changes in the composition of the blood, rather than of the external medium, became the more important stimuli to which the animal should respond, the pituitary became removed from the surface and bathed more copiously with blood. Its close connexion with the brain is curious; it would be readily understandable if the glandular part of the hypophysis was supplied with nerve fibres, but the connexion seems rather to be the other way round. In the higher animals, at least, it is the fluid draining from the brain and containing secretions from the neural tissue which reaches the glandular part (Harris, 1955). It may be that the fluid leaving the brain can be regarded as the fluid which needs corrections to be made in its composition and the anterior pituitary thus becomes the chemoreceptor for this neural effluent. Osmoregulation itself, for some reason at present unknown, appears to have been handed over to specialized nerve cells (Verney, 1947), but the gland cells proper are in a position to respond to other changes in the blood that has just penetrated through the nerve tissue, so that just as the carotid body is, in a sense, monitoring the blood going to the brain, the anterior lobe of the pituitary is monitoring it as it comes away, and instructing the homoiostatic organs in their duties accordingly. It is a point of some significance to the present discussions that the nerve cells of the supra-optic nucleus produce and pass down their processes a secretion which is first seen extracellularly and which is mucopolysaccharide in nature (Perks, 1958). The association between the adrenal cortex and the essentially nervous tissue of the adrenal medulla can perhaps be regarded as being a similar situation in reverse and as providing a feed-back mechanism for the hypothalamic-pituitary mechanism, but the true nature of this association is still a mystery.

Referring back once again to nemertines (see p. 334), it is pertinent to enquire whether the ciliated groove which reaches inwards and underlies the third lobe of the brain, is not perhaps the father and mother of all hypophyses, and is not, in fact, doing for the nemertine exactly what has just been outlined for vertebrates, namely, acting as an external sense organ with close association with neural tissue. The subneural gland of ascidians is presumably another modification of the same complex (Fig. 17.4). This organ which develops from the roof of the mouth and makes contact with a large ganglion, is tubular in character and has a ciliated opening. It has been shown to have oxytocic, vasopressor, chromatophorotrophic and gonadotrophic actions (Bacq and Florkin, 1935; Carlisle, 1951), though in the last case the target organ is apparently not the gonads directly, but only indirectly by way of the neural gland and a nervous pathway. This observation has interesting repercussions with respect both to gonadotrophic action in vertebrates and to the nature of "target organs" themselves. The subneural gland of ascidians should thus probably be considered as a member of the "coelomoduct" system.

The situation in *Amphioxus* is of special interest in connexion with the origin and significance of the hypophysis. The preoral pit, which, in the young animal, for a time, opens into the left head cavity, i.e. the coelomic space of the first segment, is a ciliated pit which is apparently concerned with passing food towards the mouth. Later it becomes Müller's organ and surrounds the deeper depression which is known as Hatschek's pit; this pit, in addition to the normal ciliated cells, has long, rod-shaped, ciliated cells somewhat reminiscent of solenocytes. This whole complex should perhaps be regarded as a specialization of the "coelomoduct" corresponding to the first segment, and de Beer (1926) has suggested that it is the phylogenetic origin of the glandular part of the pituitary. The ciliated pit has a rich vascular supply which, in view of the possible "sense-organ, hormone-producing" function of the organ, is a point of some interest.

Hatschek's nephridium, situated further posteriorly, is a diverticulum from the gut, which has many of the characteristics of a true nephridium, including solenocytes, and it probably corresponds to the "coelomoduct" of the second segment. Though there is some difference of opinion as to its origin, this organ probably develops from a group of cells first budded off from the endoderm as a separate mass, and these cells later grow back as a tube to the roof of the mouth and open into its cavity. This mode of development is exactly comparable to that seen in the development of nephridia in oligochaetes (except that the original cells derive from endoderm and not ectoderm) see p. 249) and makes the interpretation of its embryology difficult. The presence of solenocytes is strongly suggestive of nephridia, but on the other hand, Legros (1910) suggested that the nephridium arose as an outgrowth of the coelomic cavity. The fate of Hatschek's nephridium in evolution is obscure.

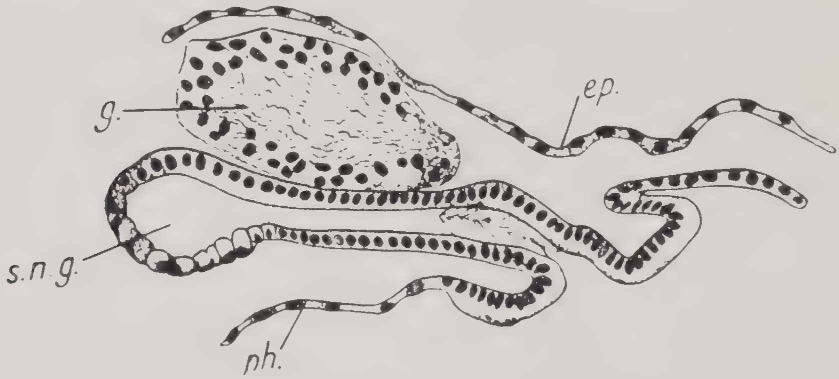

FIG. 17.4

Longitudinal section of the subneural gland of an ascidian. ep. skin; g. ganglion; ph. pharyngeal epithelium; s.n.g. subneural gland. (From Young, 1950, after Metcalf).

Whether the preoral pit and Hatschek's pit, i.e. the possible forerunners of the hypophysis, are remnants of a coelomoduct or nephridium is difficult to say, but certainly the presence of solenocyte-like cells suggests the nephridium as the more likely. On the other hand, if the composition of the fluid in the cavity determines their activity this is more in keeping with the orientation of coelomoduct cells.

One feature of the homoiostatic mechanism which is of great interest in so far as it affects the members of the "coelomoduct" complex, is the nature of the chemical mediators. It has been stressed that these mediators are likely to be by-products of normal activity, and, moreover, of the particular activity which is connected with that aspect of metabolism eventually controlled by them. For example, if substance A is actively taken in to a cell (X) it may only be possible to do so by liberating substance B from the cell (Fig. 17.5.1). Any accumulation of substance B around the cell would then presumably interfere with the uptake of substance A. Alternatively, it may only be possible to take up substance A if another substance, C, is simultaneously supplied (Fig. 17.5.2) and lack of substance C could allow A to act on another cell (Y) to make it produce C, which could in turn be used to allow the uptake of A by the original cell (X) (Fig. 17.5.3). Other similar situations can easily be envisaged.

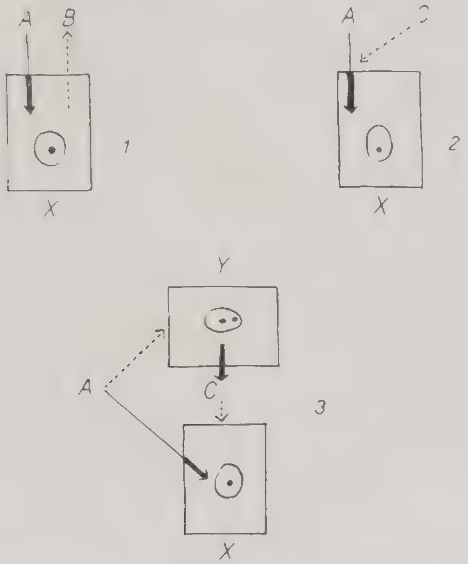

FIG. 17.5

The uptake of substance A by cell X. 1. Dependent upon simultaneous liberation of substance B. 2. Dependent upon simultaneous presence of C. 3. Dependent upon presence of C, liberated from cell Y under the influence of A. Heavy arrow, dependent process; broken arrow, necessary condition.

This is all very simplified and naive, but there is the very striking fact that the main homoiostatic hormones fall into three main chemical classes: (1) steroids, (2) mucoproteins often containing glucosamine, and (3) a group of proteins, polypeptides or even smaller molecules, rich in tyrosine, or in some way closely related to tyrosine (see Table 17.1). Many of these last also contain notable amounts of sulphur.

The Possible Significance of the Steroid Molecule in Homoiostasis

The adrenal steroid hormones fall into two main classes, those primarily concerned with tissues where the movement of Na^+, K^+ and water is paramount, and those acting on tissues in relation to their utilization or production of glucose. Among the steroid sex hormones, oestrogens (with terminal hydroxyl groups) tend to favour growth processes, at least in certain tissues. In some epithelia this entails the process of keratinization (c.f. the effects of lack of vitamin A) while glandular structures may develop in others. The male hormones (with one terminal keto group) and progesterone (with two terminal keto groups) tend to favour mucification in those cells which are capable of it, and to accelerate secretory processes (cf. excess of vitamin A). In the action of all these steroids there is bound to be much specificity determined by the minor modifications of the molecule (α or β isomers, presence of other reactive groups, and so on), and the nature of the tissue itself must also determine which steroid will fit its particular molecular pattern (Huggins, Jensen and Cleveland, 1954; Huggins and Jensen, 1954).

Table 17.1.

Classification of hormones

1. *Steroids*
    - Oestrogens [OH— ——— —OH]
    - Androgens [OH— ——— = O]
    - Progesterone [O = ——— = O]
    - Adrenal-corticoids $[\mathrm{O} = \text{———}<\begin{smallmatrix}\mathrm{OH]}\\ \mathrm{O]}\end{smallmatrix}$
2. *Carbohydrate (particularly hexosamine)—containing proteins*
    - Luteinizing hormone
    - Follicle stimulating hormone
    - Thyrotrophic hormone
3. *Tyrosine-containing (or derivative) substances*
    - Insulin (S)
    - Adrenocorticotrophic (S)
    - Growth hormone (S)
    - Thyroxin
    - Adrenalin
    - Prolactin (S)
    - Intermedin
    - Oxytocin (S)
    - Vasopressin (S)

    [Those marked (S) also contain cystine]
4. *Miscellaneous*
    - Secretin
    - Gastrin } and other gastrointestinal hormones
    - Pancreazymin
    - Parathormone

The dependence of these tissue-responses on the degree of oxidation of the end groups of the steroid molecule, suggests that the specific activity of the steroid could be related to its influence on the oxidation-reduction potential in the cells. Indeed, there is now evidence to show that some hydroxysteroids are intimately concerned in the transfer of hydrogen from triphosphopyridine nucleotide to diphosphopyridine nucleotide, provided the appropriate hydroxysteroid dehydrogenase is present (Hurlock and Talalay, 1958; Talalay and Williams-Ashman, 1958). Moreover, castration, or treatment with stilboestrol, causes a great fall in dehydrogenase and zymohexase activity and in oxygen consumption of the ventral prostate of the rat, which can all be restored to normal again by treatment with testosterone (Butler and Schade, 1958). Thirdly, suspensions of thymus cells have their oxygen consumption greatly lowered by some of the more highly oxygenated steroids, e.g. deoxycorticosterone (Jedeikin and White, 1958). It would be interesting to know at which points the steroids would have their main effects on such a scheme as that suggested in Chapter 11 for ionic transfer. There are many possibilities, but the fact that it is the end-groups of the molecules which appear to run parallel with function probably indicates that they act on some system where polarity is vitally important, as, for example, on the cell membrane. If these different steroids enter in an orientated fashion into the structure of the cell surface they may cause the cell to function very differently according to their polarity. Moreover, side groups on the main part of the molecule, or other changes affecting its shape, would then be expected to affect the ability of the individual steroids to pack into the surface, and so modify their physiological action quantitatively.

Finean (1953), Davson and Danielli (1943), Winkler and Bungenberg de Jong (1941) and others have suggested that cell membranes may be composed essentially of orientated lipids in which phospholipins predominate and in which more or less cholesterol may be incorporated. Such lipoid layers may be simple, double or multiple and may be interspersed with hydrophilic layers. Finean (1953) visualizes the phospholipin molecules as having their fatty-acid chains orientated in parallel array within the surface, the glycerol and phosphate part of the molecule being attracted to the aqueous phase of the system (e.g. the cell contents or the external medium) on account of the polar groups of the phosphate, and the choline or other base being tucked in among the fatty-acid chains (Fig. 17.6). He has suggested that cholesterol may combine with the base by the OH group in the 3-position of the steroid molecule and that the side-chain of the steroid then projects between the fatty acid chains towards the surface of the membrane. If this idea is extended to other steroids (and perhaps even to other molecules of similar shape and with a similar distribution of polar groups) a cell membrane could acquire extremely specific qualities which would depend on (a) the influence of the particular steroid molecules on the general packing of molecules on the surface; (b) the specific properties of the steroid which allow it to combine more easily with the choline of lecithin, the ethanolamine of cephalin or the corresponding reactive group of other phospholipins; (c) the specific terminal group of the steroid which would project on the side of the lipoid layer away from the phosphate groups of the lecithin etc.

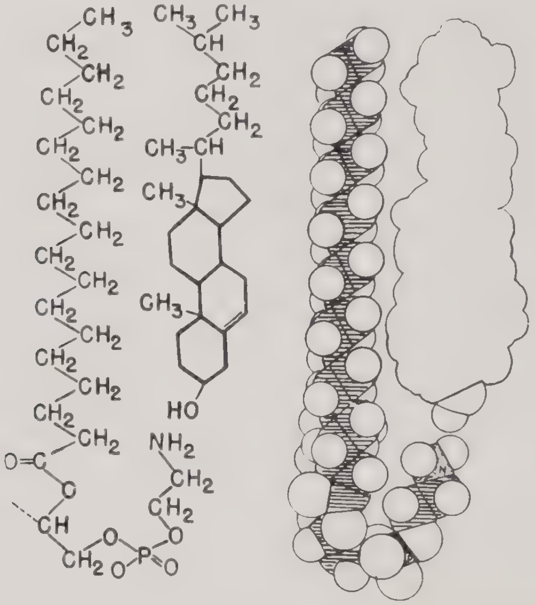

Fig 17.6

A suggestion for the manner in which cholesterol associates with phospholipins in the myelin sheath of a nerve fibre. (Finean, 1953).

In relation to the ability of the steroid to pack among the fatty-acid chains of the phospholipins the side groups of the steroid molecule would be expected to play a decisive role, and their action in this respect is likely to be much modified by the degree of unsaturation of the fatty acids themselves. As a consequence of the nature of the exposed terminal groups of the steroid molecule, the oxidation-reduction properties, the hydrophilia, the electric charge as well as the purely chemical properties of the membrane would be determined in a rather specific manner. Finally, the special location and the actual distribution of each particular steroid in the membrane would determine a surface pattern on the membrane and allow the attachment or otherwise of other reactive molecules to the general surface in a manner similar to that suggested by Gill (1959) for the way in which bisquaternary ammonium compounds may act as ganglion-blocking agents by forming stable links between their terminal quaternary groups and two reactive points on the cell surface. To take a simple case, let it be supposed that in Finean's model a certain number of the cholesterol groups are replaced by oestradiol, the actual spacing of the OH groups thus introduced in place of the cholesterol's side chain could be very important in determining what sort of molecules would be likely to associate with or become adsorbed to that surface. Mixtures of steroids presenting different patterns of O and OH groups on the outer surface of the lipoid layer or membrane could obviously give the membrane very different properties from those presenting O or OH only; thus mixtures of oestrogens and progesterone in different proportions would alter such membranes very considerably and in ways which might be very specific. Such a system could well account for the observed qualitative differences in response which result from quantitatively different mixtures of oestrogens and progesterone and for the extremely specific way in which these mixtures affect particular tissues.

Obviously the possibilities of such systems are almost infinite and any desired degree of specificity would seem to be theoretically attainable. The limits would be set by the ability of the different steroids to pack within the membrane, by the stability of the result, and by the number of possible patterns of reactive groups which could be produced on the exposed surface of the membrane. These reactive groups and the molecules which became attached or adsorbed to them would clearly be very important in determining the ability of water and ions to penetrate the membrane.

Suppose, for the sake of argument that the more oxidized steroids facilitate the movement of Na^+ ions through the cell membrane, but that the Na^+ can only move if a mucoprotein, or some substance like glucosamine, goes in the opposite direction. Although it is irrelevant to the present argument, the movement of K^+ ions is also likely to be related to that of Na^+. The two types of adrenal steroid which, it will be remembered, are being produced by cells that have been envisaged as lying in different positions on the gradient system of the adrenal cortical cords, could well act by altering the cell membrane of their target organs in such a way that the active movement of sodium could be inwards or outwards. For example, it could be suggested that the nature of the cell membrane, e.g. brush border or involutions, could be partly dependent on the stereo-chemistry of the steroid molecule, or the extent of the involutions could be greater in the presence of some steroids than of others. Thus, in the cells whose membranes are sensitive in this way, activity with regard to Na^+, both in direction and in extent, could well be dependent on the circulating steroids and the manner in which they orientate themselves in the membrane. If the Na^+ movement is, as has been suggested, linked with the movement of some substance like glucosamine, in the opposite direction, then it is easy to imagine a system being evolved, though in our present state of ignorance it is difficult to formulate it in detail, which, by using the varying amounts of steroids of the four main physiological types together with the mucoprotein or glucosamine-derivatives circulating in the blood, tissue fluid or kidney tubular fluid could regulate these main constituents of the ionic content of the body. The "tyrosine" hormones mostly affect the uptake or liberation of glucose.

However this pattern may eventually work out, the endocrine system now emerges as a homoiostatic mechanism that has grown and evolved from earlier systems which were first concerned with excretion and the homoiostasis of such simple organisms as are most nearly exemplified by the nemertines of the present day. Moreover, the lymphoid tissue emerges as an integral part of the same homoiostatic mechanism.

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CHAPTER 18

THE RETINA IN VERTEBRATES

PERHAPS of all cytological problems in vertebrate physiology, the cellular behaviour of the eye and, in particular, of the retina is one of the most intriguing and at the same time most difficult. At first sight any evolutionary approach to its study seems to be ruled out because of the absence of any phylogenetically early or intermediate stages; the eye is already a fully formed and final product in the most primitive living vertebrates. The eyes of lampreys do not differ in any fundamental way from those of the higher vertebrates, and there are no more primitive animals with eyes which could be interpreted as half-way stages in the evolution of the complete structure.

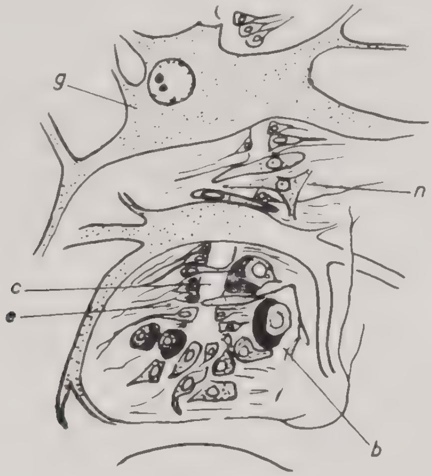

FIG. 18.1

Asymmetrically-pigmented nerve cells, "Becheraugen", in the central nervous system of *Amphioxus*.

b. Becheraugen, with asymmetrical pigment; c. Central neural canal; e. Ependymal cells; g. Giant nerve cell; n. Nerve cells (After Franz, 1923)

Amphioxus has two types of photosensitive mechanisms. It has the curiously unilaterally pigmented nerve cells in its central nervous system, the so-called "Becheraugen", (Fig. 18.1) which appear to determine the direction in which the animal swims when exposed to a source of light, as a consequence of the light stimulating these cells unequally. It also has a group of modified ependymal cells connected with nerve fibres, the infundibular organ (Fig. 18.2), which is normally shielded by an anteriorly placed pigment-spot while the animal is buried in the sand, but when illuminated, causes the animal to turn in the opposite direction, i.e. to move away from the light (Franz, 1923). Possibly this latter organ may have homologies with the eyes of vertebrates and in this connexion the fact that it is composed of flagellate ependymal cells will be seen to be of great interest. The "Becheraugen" should probably be considered as a specialization of certain nerve cells of *Amphioxus* and they probably have little connexion with the eyes of higher forms.

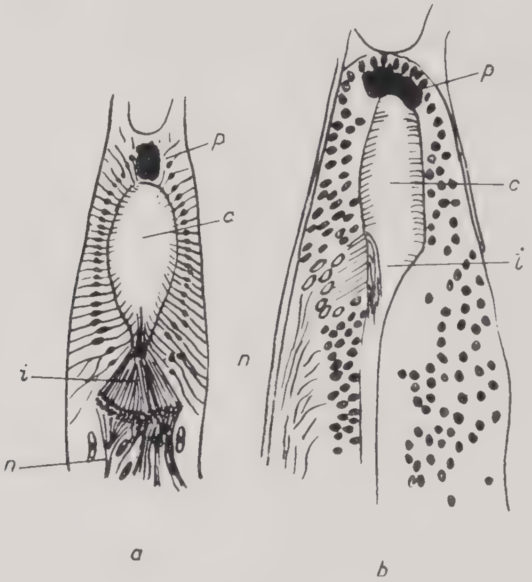

Fig. 18.2

Infundibular organ of *Amphioxus*.

(a) Vertical section; (b) Sagittal section; c. Central canal; i. Infundibular organ; n. Nerve fibres; p. Pigment spot. (After Boeke, 1913, and Franz, 1923)

A possible explanation of this apparent gap in the evolutionary history of the eye may actually lie in the quite exceptional importance of this organ to the animal. The advantages, in finding food and in avoiding obstacles and particularly predators, which accrue to an animal from any mechanism which detects the direction of light, are obvious; and as soon as any form of image can be perceived by the animal these advantages are immediately and enormously increased. Even poor form-vision, under critical conditions of hunting or being hunted, may make all the difference between survival and death. Consequently, any modifications of structure leading to the acquisition and further perfection of image-formation in the eye, and to the interpretation of that image by the brain in terms of the appropriate motor response, have quite exceptional survival value. It is therefore at least a plausible hypothesis that the acquisition of the first eye and of the requisite neural mechanism behind it that allowed an image to be formed and used, was the beginning of a very rapid process of evolution in which each step towards perfection immediately made the older models obsolete. The whole evolutionary development of the vertebrate eye may therefore have occurred with great rapidity and within a brief period of geological time owing to the enormous pressure of selection. Moreover, once the final and functional result had been achieved, there was unlikely to be any retrogression except in one or two isolated environments, e.g. in cave-dwelling where the organs seem to atrophy. Moreover, there would probably be little chance of any second mechanism developing in competition with the already more efficient mechanism, because it would always be initially less efficient and, unless it provided additional information, would be unlikely to have any survival value.

The conclusion to be drawn, therefore, from this argument, is that the eyes of all vertebrates are essentially the same; they are all modifications of a common plan. From the cytological aspect this means that the cells which contribute to the formation of the eye are basically the same in all vertebrates. It may be that the initial and essential step in eye-formation is the one already evident in *Amphioxus*, namely the development of the elongated and flagellate cells in the infundibular organ, stimulation of which by light causes movement of the animal. In other words, in *Amphioxus*, photosensitive cells are already linked with a neural mechanism so that changes induced in the photoreceptors by light can be translated into some sort of neural response, and the neural responses integrated into the performance of co-ordinated and "adaptive" movements. Thus, simple as this "eye" of *Amphioxus* appears, it does already involve considerable specialization, e.g. a photochemical reaction, the utilization of this reaction to set up a neural disturbance, the integration of this neural disturbance into the general neural pattern and the establishment of "reflex" paths to motor units. Once such a pattern has been organized in outline, first to make use of light for directional purposes, and then as an image-forming system, its future development is assured and, as suggested earlier, may have been a very rapid process and one against which other less efficient systems would be unlikely to compete. Whether the infundibular organ of *Amphioxus* itself is to be regarded as an organ that is truly homologous with, or only analogous to, the eye of vertebrates is a moot point. Its existence does, however, indicate at least the potential photosensitivity of the ependymal cells; and what has happened once in the stock of *Amphioxus*, i.e. the production of the infundibular organ from neural tube material, could well happen again, perhaps independently and somewhat differently, but from the same classes of tissue, to produce the paired photosensitive organs common to all vertebrates.

THE DEVELOPMENT OF THE EYE

Many theories have been put forward to account for the development of the eye in vertebrates, for the inversion of the retina and for the manner of lens formation in co-ordination with that of the neural mechanism. At present, there seems to be little chance of reaching any definite conclusion on this matter, but there is at least one point upon which there can be no doubt: the retinae of all vertebrates develop from the walls of the neural tube, so that, both structurally and functionally, the retina is likely to possess many features in common with other parts of the nervous system, since both are developed from the same material after it has already achieved some degree of specialization.

The embryological development of the eye may be briefly recapitulated at this point in order to indicate those features of the final structure upon which a study of their historical development seems to shed some light. The first stage in eye formation in most vertebrates is recognizable as an outward bulging of the neural tube to form an optic vesicle. The cause of this outward bulging does not yet seem to have been adequately explained, but the observations of Weiss (1934) on the fluid secreting powers of the ependyma of the neural tube of chicks, may perhaps suggest a possible mechanism. He found that ependymal cells from the roof of the neural tube become surrounded with pools of fluid in tissue cultures in a plasma and embryo extract medium, while the nerve cells of the mantle did not do so. Nevertheless the origin of this fluid is uncertain and it may have arisen by lysis of the plasma itself, in which case it would be unwise to regard fluid secretion pressure as certainly involved. Nevertheless, the neural tissue of cyclostomes and some teleosts is initially a solid cord of cells that subsequently becomes tubular, so that in these animals secretion by the lining of the tube may well be a real occurrence, and thus could also be a factor in optic vesicle formation here and elsewhere. As in other foldings which go on in development, however, the shapes of the cells may be more important than their secretory powers, although the two may not be unconnected.

At first, the cellular constitution of the optic vesicle does not noticeably differ from that of other parts of the neural tube. This is a matter of some importance because it has quite clearly been shown, on several grounds, that the neural tube sooner or later becomes a structure which is by no means homogeneous. The ventral portion or basal plate differs remarkably from the dorsal or alar plate not only in the frequency and the time of occurrence of mitoses (Hamburger, 1948), but also in the content of alkaline phosphatase (Moog, 1943) and in the potency and efficiency of each as an inducing agent for other tissue differentiations (see p. 204) (Grobstein, 1955). In the urodele it has been shown that the cells of the alar plate produce sensory and internuncial cells, while the motor cells are derived exclusively from the basal plate (Holtzer, 1951). Thus the exact situation of the outgrowing vesicle in the dorso-ventral axis of the wall of the neural tube may be a very significant factor in determining the character of the retina which subsequently develops from the vesicle.

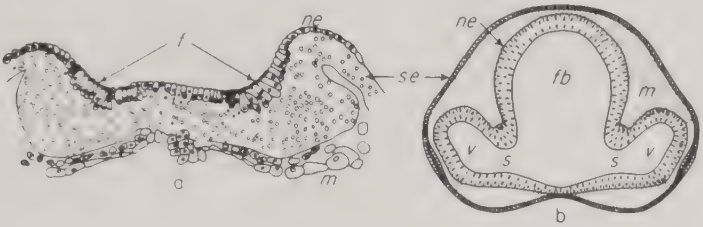

FIG. 18.3

Foveolae opticae in the frog (a) and the optic vesicles (b) which develop from them. f. Foveolae; fb. Forebrain; m. Mesenchyme; ne. Neural ectoderm; s. Optic stalk; se Surface ectoderm; v. Vesicle. (Walls, 1942)

The alar plate of course results from the fusion of the crests of the neural folds. In amphibia these crests are composed of flagellated or ciliated cells (Assheton, 1896), while the neural plate, from which the basal plate derives, has a tall columnar epithelium which, at this stage, is not visibly ciliated. This initial difference may to some extent persist and lie at the root of the other differences which arise later. In the frog, the foveolae opticae, which are thought to be the precursors of the optic vesicles, lie between the neural folds and the basal plate, with feet, as it were, in both camps (Fig. 18.3).

As the outward bulge of the optic vesicle approaches the outer surface of the body, i.e. the surface ectoderm, there develops, generally by some sort of inductive process, a thickening of the ectoderm and the formation of the first signs of a lens. The extent of the dependence upon induction and the inherent capacity of the ectoderm of this region of the embryo differ in different groups; in some, the inductive process is essential and highly localized in space and in time; in others, a lens may form in the complete absence of an optic vesicle. Other parts of the neural tube may bring about lens formation also, so the specificity of the "induction" is not always of a very high order, and in some species, particularly among the amphibia, lens formation may take place in other parts of the eye-complex in the absence of the proper lens. In fact, an important feature of eye development seems to be the inhibition of the formation of further lens tissue by the lens itself (Rose, 1957).* From the cytological point of view there are many interesting and puzzling features about lens formation that will be considered later, but it is noteworthy that the lens very early develops a vesicular structure which subsequently "invaginates" (Fig. 18.4) and it is only the cells of the inwardly-directed hemisphere that contribute to the formation of the actual "lens-fibres", which are themselves cellular or intracellular. In some respects the lens, in its early stages, resembles a little blastula in which the blastocoele cavity is eventually obliterated by the elongation of the cells of the hemisphere in contact with the inducing optic vesicle. This elongation of the cells, which in the chick depends on the direct contact between lens epithelium and optic vesicle without any intervening basement membrane or other tissue (Fig. 18.5) (McKeehan, 1951), though a sulphated mucopolysaccharide

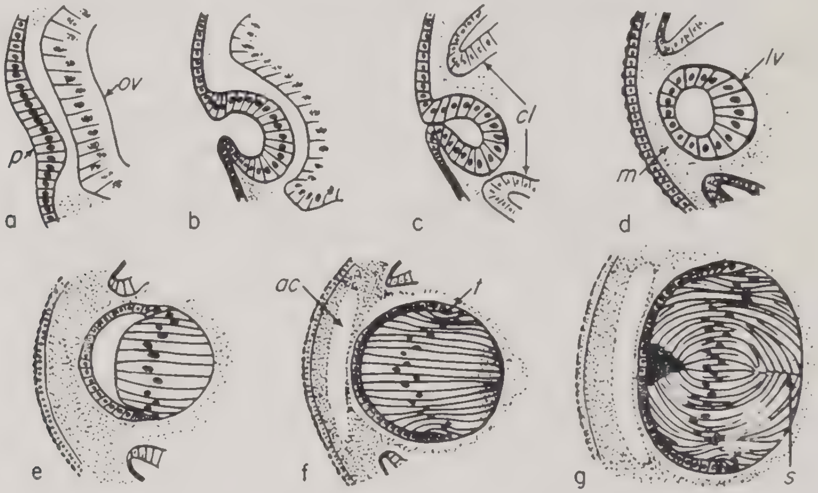

FIG. 18.4

Changes in the epithelium over the optic vesicle which lead to the formation of a lens. Note the blastula-like arrangement of cells in d. and e.

ac. Anterior chamber; cl. Lips of optic cup; lv. Lens vesicle; m. Mesenchyme; ov. Optic vesicle; p. Primordium of lens; s. Suture; t. Transitional zone. (From Walls, 1942. After Mann, 1928) may be detected between the two (Smelser, 1956), is accompanied by the intracellular accumulation of the lens proteins—α and β crystallin.

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* See note on page 392.

After the induction of the lens, the optic vesicle itself begins to collapse inwards and withdraws from contact with the lens. The induction of the primary lens, if caused by the optic vesicle, is presumably the result of exudations from it, possibly of a polysaccharide, or is caused by stereochemical influences dependent on the molecular structure of the outer wall of the neural tube, or by the removal of some substance from the environment of the surface epithelium by the neural cells, to which removal the epithelial cells respond by the change towards lens formation.

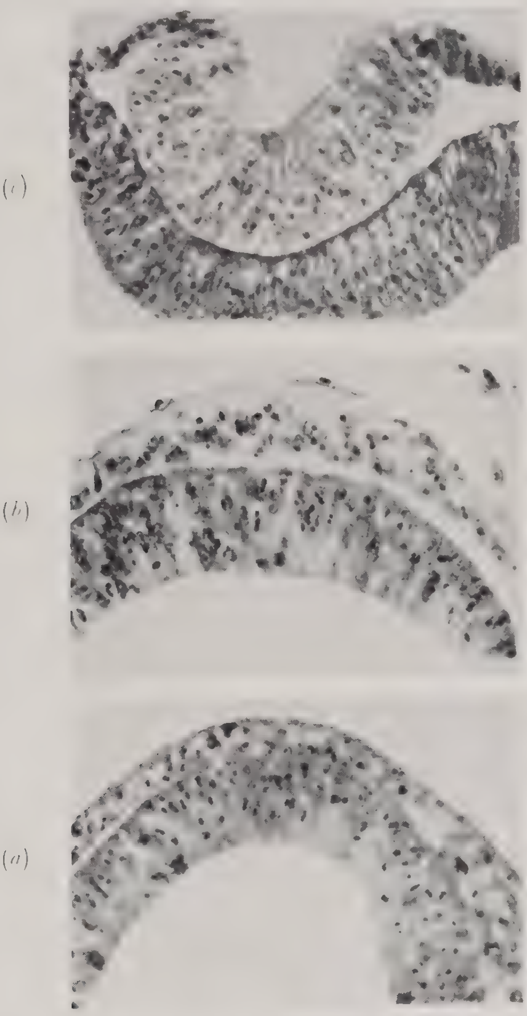


Fig. 18.5

Three stages in the development of the lens from the surface epithelium and the parallel changes in the optic vesicle. (McKeehan, 1951)

In most areas elsewhere, the outer surface of the neural tube becomes covered first with pia mater cells and then with the mesenchymatous dura mater. The pia mater cells are thought to emanate from the region of the neural crest and to spread over the neural tube, but it is noteworthy that, in the adult eye at least, the tissues derived from the layer of cells corresponding to the pia mater are missing from the region of the eye lying immediately between the lens and the retina. The cells of the pia mater in the eye, i.e. those cells which are comparable in origin with those elsewhere eventually covering the neural tube and forming the pia mater, contribute to the pigmented cells of the choroid coat and to the pigment cells in the substance of the iris, though not, of course, to the actual pigmented epithelium of the iris, whose origin is akin to that of the retina itself. The lens therefore forms where this incompleteness of the pia mater as a covering of the neural tube exists, and where the neural tube cells can have direct contact with the overlying ectoderm.

The nature and cause of these curvatures which develop in the formative epithelia of the lens and the retina are still largely unknown. The development of cell polarity, the relative movements of fluid, changes in ionic content, changes in water content, alterations of cell shape, and molecular interactions occurring at the surfaces of the cells all suggest themselves as possibilities, but the true nature of retinal invagination and the cause and delineation of the field for lens formation are still matters for investigation. The cavities of the brain and the neural canal as a whole all undergo such a remarkable reduction in size at about the same time, that an absorption or removal of fluid from the neural tube must occur and could be a contributory factor, but the very complexity of the subsequent differentiation of the different regions of the optic vesicle and other parts of the central nervous system argues against any one such simple cause, and yet the final pattern achieved is a simple one, the eye being to all intents and purposes spherical. The biological advantages which follow the invagination of the optic cup, and the consequent formation of an ideal surface upon which the cornea and lens can focus an image, are obvious, and, in a sense, could be regarded as explanatory, but the physiological mechanism which brings them about is quite another matter.

The in-pushing of the optic vesicle results in the formation of the two-layered optic cup (Fig. 18.6), and the destination and potentialities of the cells in the different parts of this cup are matters of great importance in appreciating the characteristics of the retina and its associated structures. The essential cell layers are shown in Fig. 18.7.

The epithelium of the primary neural tube normally gives rise to neuroblasts, neuroglia and ependyma. In the region of the optic cup it gives rise to pigment epithelium, the epithelio-muscular tissues of the iris, a secretory epithelium over the ciliary processes and then to the rods and cones of the eye with their associated bipolar cells, horizontal cells, amacrine cells, ganglion cells and cells of Müller's fibres. This is a remarkable series of potentialities to be displayed by one comparatively small area of epithelium, but in spite of evident diversity, certain common and unifying features can also be discerned. Moreover, the development of the eye has to be viewed in relation to the behaviour of the cells lying in the immediate vicinity, namely the adjacent parts of the nervous system and the neural crest. The latter tissue certainly contains cells which are endowed with multiple potentialities.

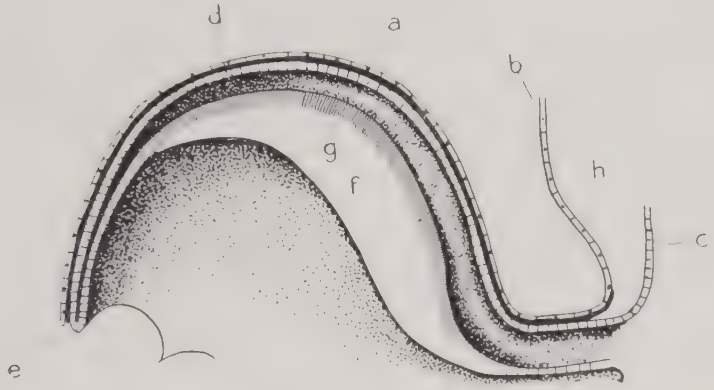


Fig. 18.6

Diagram showing the origin and development of the retina from the optic vesicle.
a. Choroid; b. Pia mater; c. Ependyma; d. Pigment layer; e. Margin of iris; f. Pars optica retinae; g. Sensory cells; h. Neural tissue. (Willmer, 1949)

The development of the pars optica retinae forms a convenient starting point from which to orientate an analysis of the peculiarities of the cytology of the eye and retina. Events occur in this region of the retina which are closely parallel to those occurring in other parts of the central nervous system. The originally more or less columnar epithelium gives rise by mitosis, to several layers of cells which much resemble in pattern the marginal and mantle layers of other parts of the neural tube (Fig. 18.8). The first cells to separate, which move furthest from the lumen, constitute the ganglion cells, the later ones form the bipolar cells, horizontal cells and amacrine cells. Those left adjacent to the original lumen of the neural tube differentiate into the visual cells proper—the rod and cone cells. Most of the cell divisions appear to take place among the cells which are actually adjacent to the lumen, but it is probable that this is an artefact and that cells of other layers whose nuclei are well removed from the lumen, can also divide, but before doing so they move towards the lumen as though by retraction of processes still connecting them with the surface (Watterson, 1956).

The Nature of Rods and Cones

The key problem with regard to the retina obviously lies in the nature of the rod and cone cells and their relationship with one another. They are cells which, relative to the neural canal, are exactly comparable in position with the cells of the ependyma and choroid plexus. The first sign of their differentiation in the eye is in the production of fine processes or flagella and a bulging of the cell into the lumen beyond the terminal bars of the epithelium (Mann, 1928). Into this bulge there is a migration of mitochondria (Leplat, 1913; Lewis, 1923).

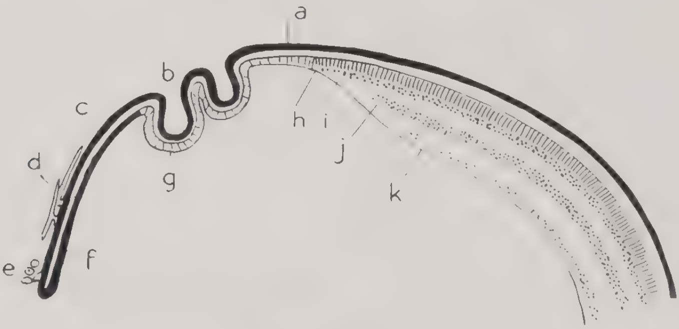


Fig. 18.7

The cell layers in the developing retina and associated structures.

a. Pigment layer
b. Ciliary processes
c. Pigment layer behind iris
d. Dilator pupillae muscles
e. Sphincter pupillae muscles
f. Pigment layer behind iris
g. "Glandular" cells of ciliary processes
h. Rods and Cones
i. Nuclei of rods and cones
j. Nuclei of bipolar cells
k. Nuclei of ganglion cells

(Willmer, 1949 and 1951).

It will be remembered that the ependyma is characterized by cells with flagella, and recent work with the electron microscope leaves very little room for doubt that the outer segment of a rod is, in fact, a modification of a flagellum (de Robertis, 1956; Sjöstrand, 1953 a and b). In the rod of the guinea-pig, and of those other species which have been examined, there is a fibre process which joins the inner and outer segments and this process has, in transverse section, a pattern very like that of all cilia and flagella, namely the nine sets of peripheral fibres, but the two central fibres (Fig. 18.9) have not so far been detected. In the inner segment this ends in a typical basal body and a

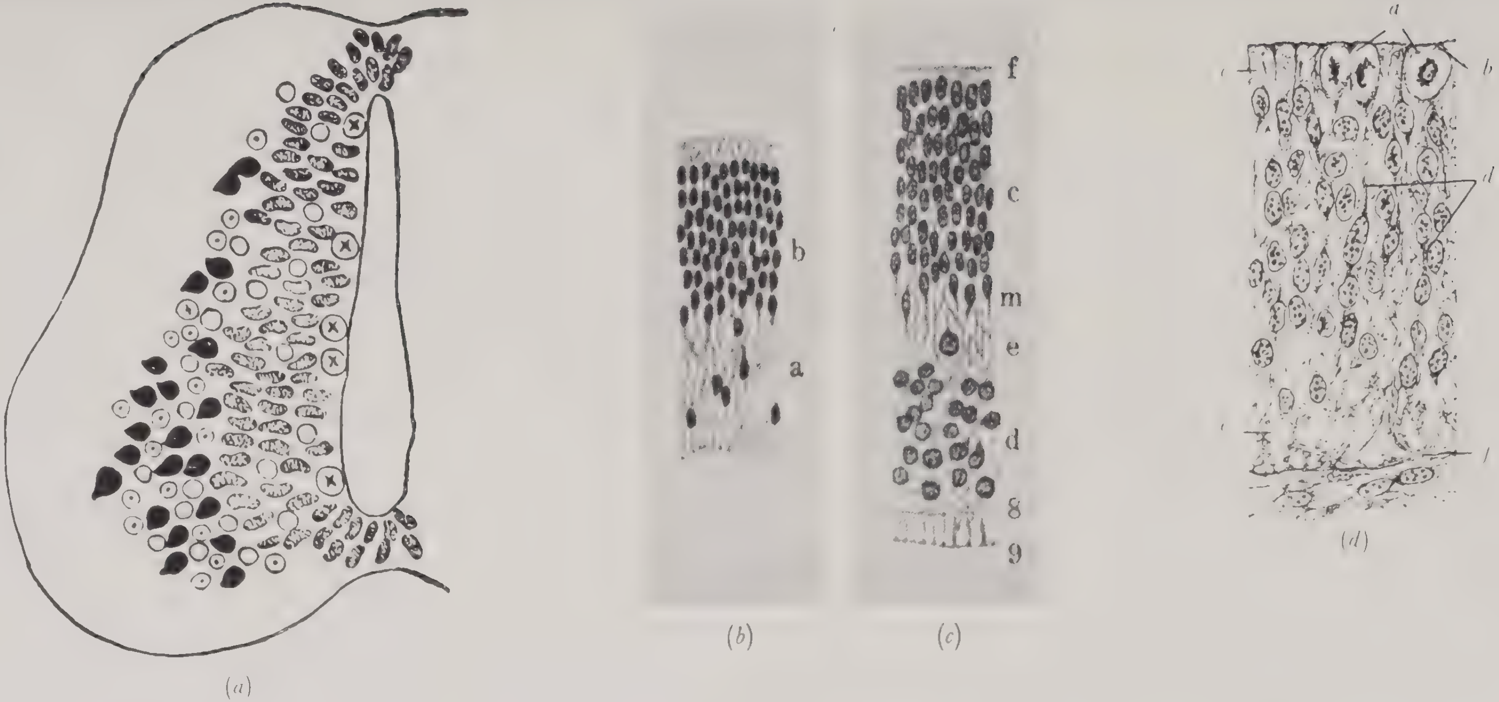


Fig. 18.8

A comparison between the cell layers in the developing retina and those in the C.N.S. (a) Development of cells in the neural tube; ⊗ Dividing cells. ○ Indifferent cells. Germinal cells. ⊙ Neuroglia cells. ● Neuroblasts (From Kuntz, 1946).
(b) Developing layers in the retina (12 mm human embryo). a. Marginal layer; b. Primitive neuroepithelium (From Detwiler, 1943).
(c) Developing layers in the retina (17 mm human embryo). c. Outer neuroblast layer; d. Inner neuroblast layer; e. Transitory layer; f. External limiting membrane; m. Müller's fibres; 8. Nerve fibre layer; 9. Internal limiting membrane (From Detwiler, 1943).
(d) Neural tube of 7 mm pig embryo. a. Dividing cells; b. Internal limiting membrane [= External limiting membrane of retina]; c. Ependymal layer; d. Mantle layer; e. Marginal layer; f. External limiting membrane [= Internal limiting membrane of retina] (From Larsell, 1939).

further cross-banded root fibre has also been described as running towards the cell nucleus, as in other ciliated and flagellate cells. The parallel plates or lamellae, that finally constitute the rod proper, develop from vesicles which arise in the "sarcoplasm" between the fibrils of the flagellum (Fig. 18.10). These vesicles are at first arranged longitudinally, but they progressively reorientate themselves and finally lie at right angles to the axis of the rod and, at the same time, become flattened into double-walled flat plates that are piled one on top of the other (de Robertis, 1956). The rod itself therefore can be considered as a modified flagellum and the whole rod cell can thus legitimately be regarded as a specialization of the typical flagellated ependymal cell; the analogy or possible homology with the cells of the infundibulum of *Amphioxus* thus springs clearly to mind and the association between flagellated cells and photosensitivity is again evident (see pp. 168, 359).

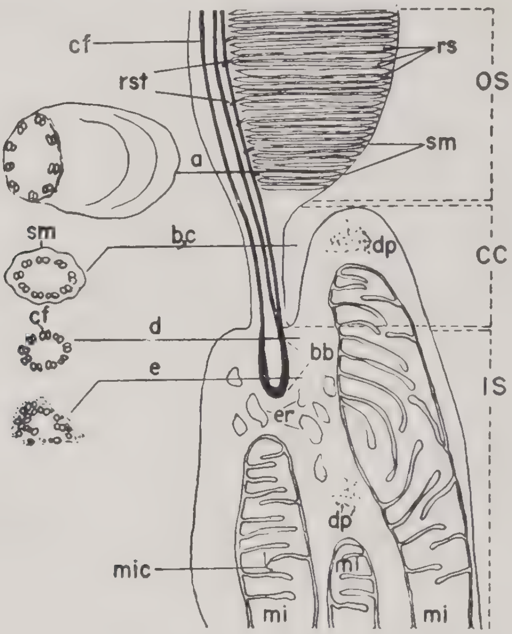


Fig. 18.9

A diagram showing the chief structures at the junction between the inner and outer segments of a rod.

OS, Outer Segment; CC, Connecting Cilium; IS, Inner Segment
a-e, Positions of sections
bb, Basal body; cf, Ciliary filaments; dp, Dense particles; er, Endoplasmic reticulum; mi, Mitochondria; mic, Mitochondrial cristae; rs, Rod sacs; rst, Tubule from rod sac to fibril; sm, Surface membrane. (de Robertis, 1956).

The development of the cones has been described along very similar lines and probably the outer segment of the cone is somewhat similar. However, attention may be called to the fact that the choroid plexus, another specialized region of the ependymal epithelium, has cells with two types of process facing the lumen, flagella or cilia in some cells and polypoid microvilli in others (Millen and Rogers, 1956)

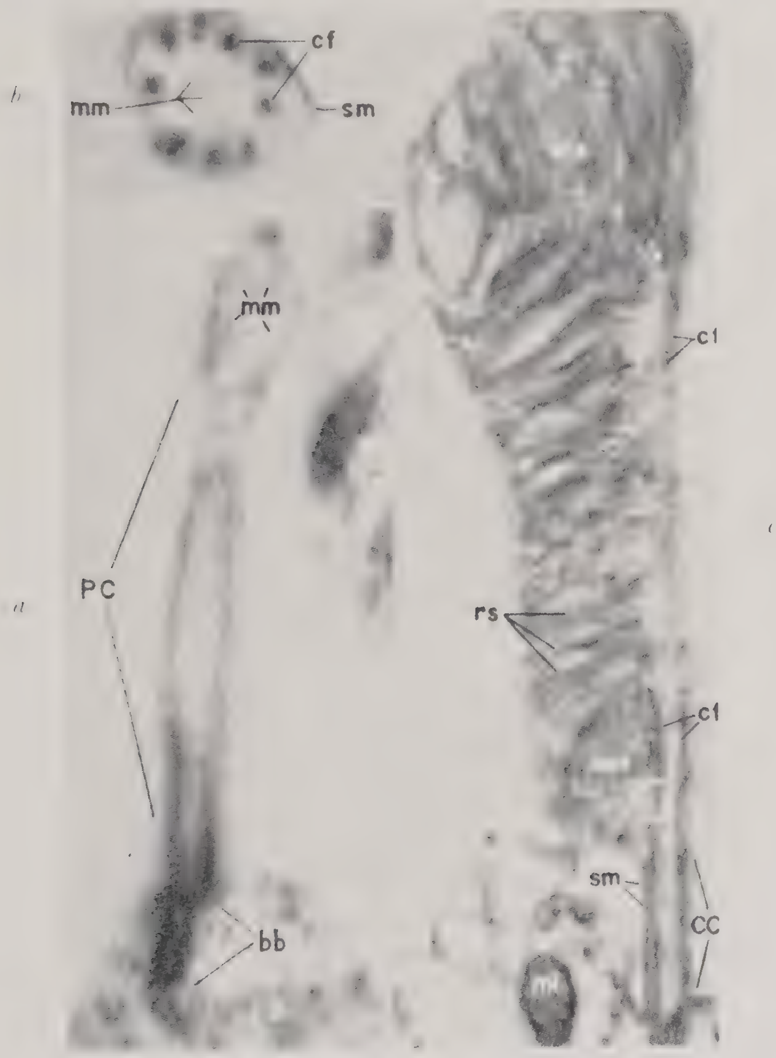


FIG. 18.10

Electron-microscope figures showing the fibres of the connecting process and outer segment of the rod and the vesicular structures which give rise to the laminae in the mouse.

(*a*) Primitive cilium (PC) with basal body (bb) and 'morphogenetic material' (mm). Inner segment (IS) (8-day mouse).

(*b*) Transverse section of cilium, with filaments (cf) and 'morphogenetic material (mm) within the surface membrane (sm)

(*c*) Outer segment from 12-day mouse. cc, connecting cilium; cf, filaments; mi, mitochrondrium; rs, rod sacs; urs, unorientated rod sacs. (de Robertis, 1956).

(see Fig. 12.2), so that perhaps a somewhat similar difference may exist in the potential retinal cells. In one of the few animals where the electronmicroscopic structure of the cones has been investigated, namely the perch (Sjöstrand, 1953a), the arrangement of the laminae in the outer segment of the cone is certainly different from that in the rods of the same animal. In the rod the laminae are double structures, i.e. flattened vesicles; in the cones they appear to be single membranes and thus have presumably not developed from collapsed vesicles, as in the rods (Fig. 18.11). A recent finding by Moody (1959) indicates that the laminae of the cone in the frog are indeed infoldings of the surface membrane of the cell. Moreover, it is probable on functional grounds that the detailed structure of rods and cones must be different, because light falling obliquely on the cones is a far less efficient stimulus than light falling axially down the cone itself (Stiles and Crawford, 1933), while the direction of the incident light makes very little difference to the performance of the rods (Flamant and Stiles, 1948). The refractive indices of the rod (μ = 1·41) and cone (μ = 1·39) outer segments are certainly different and this in itself may account for some difference in directional effect, though the basis for the refractive index difference is not established (Sidman, 1957). Cones have about 30% of solids whereas rods have over 40%. The facts that the cone laminae are single, and probably not directly comparable with the flattened vesicles of the rods, and that the laminae do not show the incision which each rod leaflet shows, possibly indicate that while the rod seems undoubtedly to be a modified cilium or flagellum, the cone could be a modified microvillus of a type similar to those seen on some of the cells of the choroid plexus. The cells of the infundibular organ in the elasmobranch, as compared with those of the same organ in

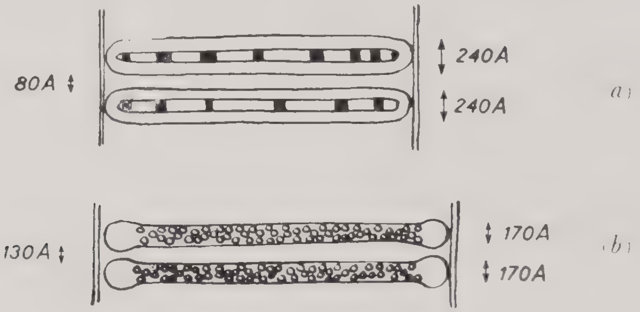


FIG. 18.11
Comparison between the arrangement and structure of (*a*) the rod laminae (guinea-pig) and (*b*) those of the cone laminae (perch). (After Sjostrand, 1953).

Amphioxus form an interesting comparison on similar lines (Fig. 18.12) (Boeke, 1913). On the other hand, the observations of Mann, (1928) on the development of the human cones point to their origin from flagellate cells in a manner very similar to that of the rods. The arrangement of the basal granules of the developing cones, however, differs from that of the rods. Usually there are two basal granules at the base of the cone process, and they lie in a plane parallel to the external limiting membrane. Thus there is some suggestion (Fig. 18.13) that the cone process may not be a modified flagellum, but perhaps may develop from something more akin to the microvillus.

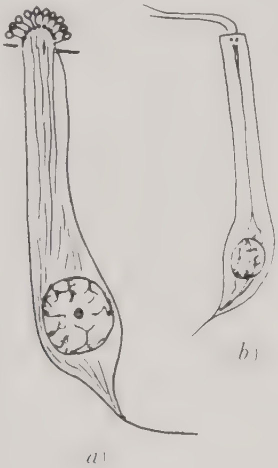


Fig. 18.12

A cell of the infundibular organ of an elasmobranch (*a*) compared with one from *Amphioxus* (*b*). (After Boeke, 1913).

If, as suggested earlier, the lining of the primitive neural tube is organized for maintaining the ionic stability of the cerebro-spinal fluid, it may well do so by having cells orientated in opposite directions with respect to ionic movement and related properties. The recent work of Bowsher (1957) on the location of serum proteins labelled with radioactive sulphur after their injection into the ventricles, indicates the absorptive and perhaps regulatory function of the ependyma. The injected protein was located in the ependymal cells and also in certain tufts of cells in the choroid plexus, but not in all the cells of the plexus. This could be taken to mean that the ependymal cells and some cells of the choroid plexus were capable of absorbing protein, while the other cells of the plexus were not.

In some ways a system of opposing polarities is what the rod and cone cell layer of the retina seems to possess. There is more than a suggestion of functional antagonism between rods and cones (Granit, 1947; Granit and Tansley, 1948; Gernandt, 1947) and there are also other features of the epithelium of the optic vesicle which suggest

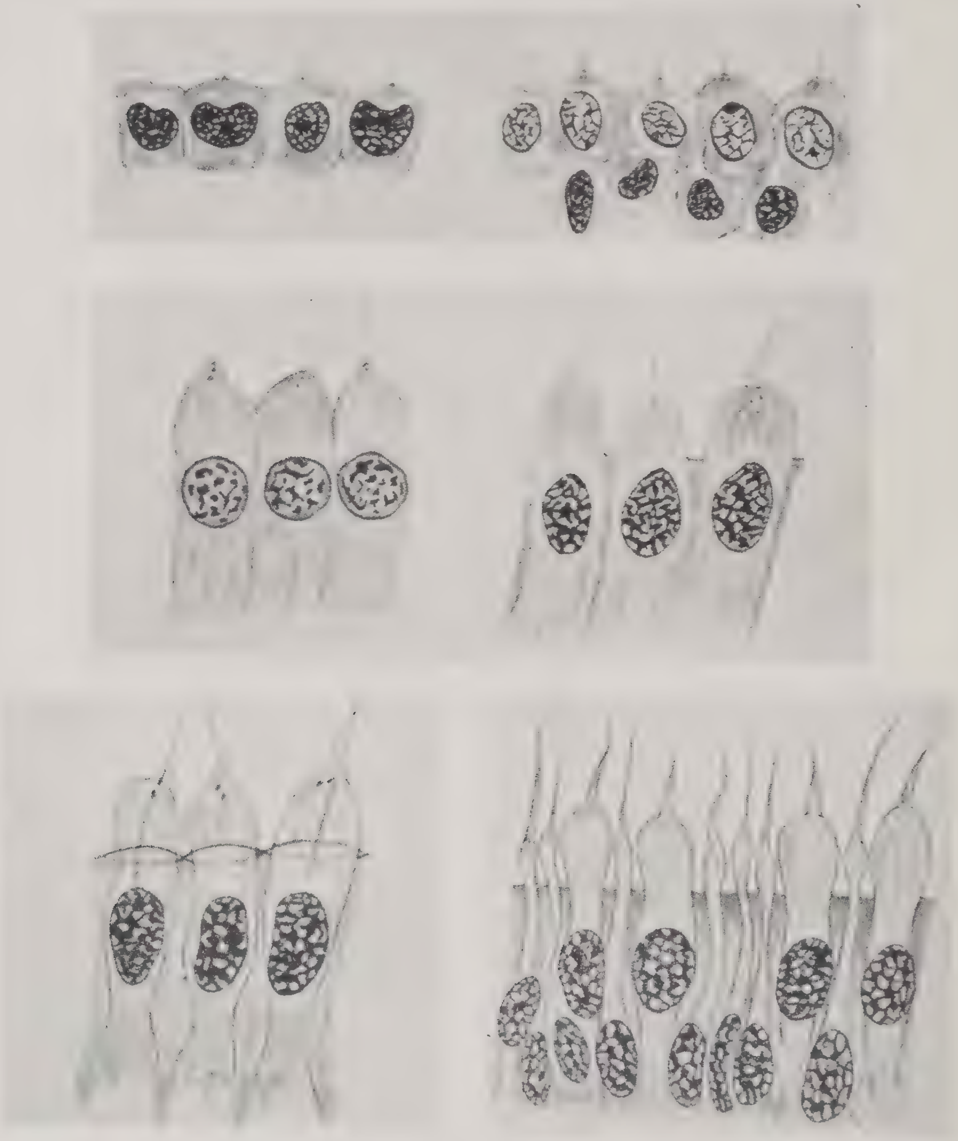
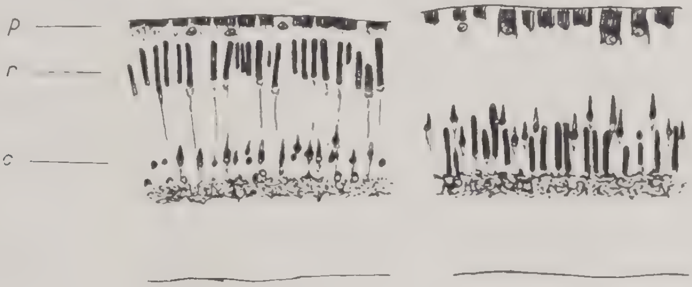


FIG. 18.13
Stages in the development of the rods and cones in the human eye.
(From Detwiler, 1943, after Mann, 1928)

that a similar duality occurs throughout the whole tissue and which, at the same time, emphasize the common properties of this epithelium as whole. In the first place, photosensitivity itself is not rigidly confined to the rods and cones; it may manifest itself in the form of photomechanical responses in several of the other tissues derived from the epithelium of the optic vesicle. Even the rods and cones may show such movement. When light shines on the eye of the catfish the cones shorten their inner segments but the rods lengthen theirs (Fig. 18.14). In other species, similar effects are frequent in either one group of photoreceptors or the other. They seldom occur in both, but when they do, they are in opposite senses (Detwiler, 1943). The antagonistic sphincter and dilator pupillae muscles are directly derived from the epithelium of the optic vesicle at the back of the iris (see Fig. 18.7). In elasmobranchs, eels and frogs, the sphincter pupillae responds directly to light in the absence of either nerves or hormones (Brown-Séquard, 1847; Weale, 1956). The dilator pupillae in elasmobranchs is controlled by nerves (Young, 1933). The response to light of these two muscles is always opposite, and in most animals, where both muscles are under the influence of either hormones or nerves or both, the muscles still remain antagonists and generally give opposite reactions to acetyl-choline and adrenalin. In this connexion, it is interesting that in the selachian, where the sphincter pupillae is directly light-sensitive, it is not sensitive to either adrenalin or acetyl-choline, whereas the dilator pupillae is sensitive to both, and both cause contraction. Other cells of the optic vesicle are also sensitive to the autonomic transmitter substances, i.e. adrenalin or acetyl-choline. For example, the cones of one class in the frog shorten under the influence of adrenalin while the pigment cells of the retina expand their pigment under its influence, if the animal is in the dark-adapted



(a) (b)

FIG. 18.14

(*a*) Light-adapted and (*b*) dark-adapted retina of the cat-fish (*Ameiurus*), showing almost complete reversal of the positions of the rods and cones. In the light-adapted state the rods are elongated and situated near the pigment epithelium, the cones are shortened.

c, Cones; p, Pigment cells; r. Rods. (After Detwiler, 1943) condition. There is some evidence that in the light-adapted condition the movement of the pigment may be reversed by adrenalin (Dubois-Poulsen, 1937), a paradoxical effect which is not unlike those seen elsewhere with adrenalin, as for example on the stomach musculature. Adrenalin has no visible action on the rods of frogs, but acetyl-choline, choline and pilocarpine are said to cause an increase in their visual purple content (Ayres and Kühne, 1882; Hwang and Hosoya, 1950).

There is no evidence as yet for any chemical transmitter-substance between the rods and cones and the bipolar cells. However, the synaptic layer between the bipolar cells and the ganglion cells, though it is somewhat further removed from the primary epithelium, is heavily charged with cholinesterase, suggesting that acetyl-choline may be instrumental as a transmitter-substance in at least some of the synapses in this layer (Anfinsen, 1944; Francis, 1953; Hebb, Silver, Swan and Walsh, 1953).

Evidence obtained by electrical recording of the impulses set up in optic nerve fibres of the retina of animals, suggests also that there is an antagonism between rods and cones in the sense that a great many effects are explicable if inhibition of certain forms of rod activity is caused by cone activity. For example, in pure rod retinae or in other eyes when only rods are in action, most of the ganglion cells give pure on-effects, i.e. the impulses set up in the optic nerve coincide with the duration of the light stimulus. Eyes in which cones are numerous give much more complex responses, and inhibition by the cones would explain many of them (Granit, 1947). It should, however, be emphasized that inhibition can also be brought about by other means and can occur when only rods are functioning (Donner and Willmer, 1950). In other types of eye also, for example *Limulus*, where it is probable that there is only one type of receptor, lateral inhibition (i.e. inhibition which is caused by neighbouring elements) has been clearly demonstrated (Hartline, Wagner and Ratliff, 1956; Hartline and Ratliff, 1957).

Many of the properties of rods and cones are naturally very similar, as one would expect them to be in accordance with their having developed in a very circumscribed area of the embryo and having acquired the common property of photosensitivity, but there are sufficient differences between them to justify the belief that they really represent two rather distinct races of cells, or at least that they are cells showing two rather different phases of activity. The relationship between them is in some ways not unlike that between the mechanocyte and the amoebocyte and basically, it may have a similar origin. A closer comparison may perhaps be that with oligodendrocytes and astrocytes, and indeed this receives some further support by the fact that, when the retina and the iris muscles of the chick embryo are grown *in vitro*, small bipolar cells, comparable in appearance and behaviour with oligodendrocytes under the same conditions, emerge in great numbers from the explants (Meyer, 1936 a and b). In view of the action of drugs on the cells in question, the relationship may also be compared with that of sympathetic and parasympathetic nerve cells. Table 18.1 illustrates the common characteristics and also the differences.

TABLE 18.1

A COMPARISON OF RODS AND CONES

Similarities

1. Photosensitivity: dependent on carotenoid proteins, i.e. Retinene$_1$ or Retinene$_2$ in combination with a specific opsin.
2. Photomechanical changes.
3. General structure, e.g. inner and outer segments extending beyond the external limiting membrane.
4. High succinic dehydrogenase activity associated with ellipsoids.
5. Common origin from "ependymal layer" of neural tube.

Differences

Feature	*Rods*	*Cones*
Outer segment	Rod-like	Conical (usually)
	Double membranes	Single membranes
	$\mu = 1.41$ circa	$\mu = 1.39$ circa
Pigment	Rhodopsin, porphyropsin etc. i.e. rod 'opsin'	Iodopsin, cyanopsin etc. i.e. cone 'opsin'
Inner segment	No oil globules	Oil globule frequent
	May lengthen in light	May contract in light
	Seldom with paraboloid	Paraboloid frequent
	No visible carotenoids other than photosensitive pigment	Xanthophyll, astaxanthin etc. sometimes present
Nuclei	Usually distant from external limiting membrane	Superficially placed near limiting membrane
	Nucleolus inconspicuous	Nucleolus conspicuous
Central connexions	'Bouton'	'Pedicle'
	Sometimes exclusively with mop bipolar	Sometimes exclusively with midget bipolar
	Forming large receptive fields for high sensitivity	Forming small receptive fields for high acuity
Reaction to injected iodoacetate	Rapid degeneration	Delayed degeneration
Retinitis pigmentosa	Rapidly attacked	Resistant
Reaction to adrenalin	Not evident	Inner segment may contract
Stiles-Crawford effect	Not present	Present
Association with pigment epithelium	With less pigmented cells	With more pigmented cells

CAROTENOIDS AND THEIR DISTRIBUTION

By far the most characteristic property of both rods and cones is, of course, their photosensitivity, and this depends on the presence of photosensitive carotenoid-proteins. The carotenoid appears generally to be either retinene$_1$ or retinene$_2$; these are the aldehydes of vitamin A_1 and A_2 respectively. Combination of these prosthetic groups, or variants of them, with proteins (opsins) from rods or cones leads to the formation of all the various photopigments that have so far been found in vertebrates. The exact character of the proteins and the steric configuration of the chromophore group (Hubbard and Wald, 1952) both alter the absolute photosensitivity and the spectral sensitivity of the pigments concerned. For these reasons, therefore, there may be an almost unlimited series of pigments in different species, yet all may be fundamentally similar in that they are carotenoid pigments combined with a protein. It is significant for this argument which seeks to differentiate between rods and cones, that when protein extracts of predominantly cone retinae are made to combine with the correct isomer of retinene (Wald, Brown and Smith, 1952, 1953) the resulting spectral sensitivity is different from that obtained by combining the same retinene with the proteins extracted from predominantly rod retinae. This clearly shows that the proteins in the two types of cells are different.

The whole area of neural epithelium in the region of the eye is specially concerned with carotenoids and it can induce them to form photosensitive chromoproteins; but the cells fall into two classes in respect of the resulting compounds. Two questions arise at this point: first, how local is this "interest" in carotenoids; and second, what is the cause of their presence? Apart from the interstitial cells of the gonads and related structures (see p. 303) carotenoid pigments occur in small amounts in the epidermis in general, in some fat depots, in the liver, in the muscles of some fish, and otherwise only in the chromatophores, though the epithelium of the alimentary canal is able to alter and metabolize them to some extent. They must always be supplied in the diet, at any rate in the higher animals.

Broadly speaking, however, carotenoids accumulate in quantity only in the eye, in the chromatophores and in the cells derived from the "coelomoducts", as for example the adrenal cortex and the interstitial cells of the gonads. Of these, it is now believed that the chromatophores are all derived from the neural crest tissue which, as has already been pointed out, is embryologically not far removed from the presumptive retinal tissue, and thus, in this respect, could be regarded as forming part of the same system of cells as the cells of the retina.

A comparison between the properties of chromatophores and certain cells of the retina is therefore not without interest. Table 18.2 shows some of the main similarities and differences.

It is thus clear that in this region of cells at the level of the neural crest and the optic vesicle, melanin formation, carotenoid storage and the related property of photosensitivity, are peculiarly developed. Moreover, the neural crest cells contain among them the cells that give rise to the parasympathetic and sympathetic ganglia, to dorsal root ganglia and to neurilemma cells. In this connexion the sensitivity to adrenalin and/or acetyl-choline on the part of the pigment epithelium, the ciliary epithelium, the sphincter and dilator pupillae muscles, the rods and cones, and, on the basis of positive tests for cholinesterase in the appropriate synaptic layer, of some at least of the ganglion cells is also of some interest. All examples of adrenalin producers elsewhere in the body are ultimately derived from neural crest tissue. This tissue, as discussed on p. 227, has a predilection for a special form of tyrosine metabolism, so that either melanin or adrenalin is commonly produced. The formation of melanin itself is, of course, well known to be increased by the action of light. It will also be remembered that in tissue cultures of neural crest, the appearance of melanin in the cells is dependent on the presence of phenyl-alanine in the medium. Furthermore, phenyl-alanine when fed to cultures of embryonic ventral ectoderm causes certain cells to develop melanin granules though this tissue normally has no interest in melanin and never forms it. The "melanin metabolism" therefore can be considered at this stage as being induced or triggered off by phenyl-alanine. Phenyl lactic acid is ineffective and indeed prevents melanin formation (Wilde, 1955). In this respect, therefore, it appears that phenyl-alanine can be considered as a kind of inducing agent for one phase of neural-crest differentiation, though its action may be partly that of supplying the right substrate; this however, is not so easily reconcilable with the action of phenyl-alanine on the ventral ectoderm, unless normally there is a high local concentration of this substance found in the neural crest at the time of its differentiation.

The observations of Figge (1940) (p. 229) on the occurrence of melanin in the melanophores and connective tissue cells of *Amblystoma* are also very relevant to this discussion and possibly throw a rather different light on the situation. They emerged from the earlier observations of Lewis (1932) that amphibian larvae do not develop pigment if grown in solutions of phenol indophenol (1:500,000). Figge concludes that this effect is caused by a change in the oxidation-reduction potential of the cells away from zero, which is near the optimum for

TABLE 18.2

	Iridophores	Melanophores	Xanthophores	Erythrophores	Cones	Rods	Sphincter pupillae	Dilator pupillae	Pigment cells	Retinal Tapetal cells
Melanin		+							+	
Guanine	+									+
Xanthophyll . .			+	(+)	+		(+) (yellow globules)		+	
Astaxanthin . .			+	(+)	+					
Vitamin A_1 or A_2					+	+			+	
Photosensitivity . .		+			+	+	+		(⊖)	(+)
Adrenalin-sensitivity . .		⊕			+			+	⊖	
Intermedin-sensitivity . .		⊖								
Acetyl-choline-sensitivity . .	(+)	(⊖)				(+)	+			
O.1M NaCl . .		⊖	⊕							
O.1M KCl . .		⊕	⊖							

+	indicates one or more definitely established cases
(+)	indicates that the evidence is dubious
⊖	= expansion of pigment
⊕	= contraction of pigment

the tyrosinase activity. What is, however, even more interesting is that he found that certain connective tissue cells (? fibroblasts = mechanocytes), which normally do not form pigment at all, can be induced to do so by treatment with phenol indophenol. He attributes this to a change of the oxidation-reduction potential of the cells towards the tyrosinase optimum, i.e. towards zero. These observations therefore have the dual interest of first indicating that melanin formation may be subsidiary to local conditions within the cytoplasm, and second emphasizing yet another difference, namely a difference in the normal balance of their oxidation-reduction systems, between fibroblasts (mechanocytes) and amoebocytes since the dermal pigment cells have many of the properties of the latter.

The occurrence of the carotenoids in the chromatophores and in the cells of the retina is one of the most fundamental problems in the physiology of vision. Wherever light-sensitivity is found throughout the plant and animal kingdoms, carotenoids or carotenoid proteins seem to be involved, and in the eyes of vertebrates vitamin A and its immediate associates are the chief participants. It will therefore be relevant to examine some of the places where vitamin A acts in order to see if there is any connexion between its other known physiological properties and its action in vision.

Vitamin A

In tissue cultures a liberal supply of vitamin A causes certain epithelia, e.g. the skin from the back of the chick, to change from a keratinizing, feather-forming epithelium to an epithelium consisting of mucus-secreting and ciliated cells (Fell and Mellanby, 1953; Weiss and James, 1955). The essential point about this change is that the epithelium changes from one which relies mostly on a passive impermeability, to one which is probably engaged actively in maintaining a proper balance of water and ions across its surface (see p. 78). The mucus-secreting cells are of the goblet type and the mucoprotein accumulates within the cells before being liberated. Conversely, rats when deprived of vitamin A, show changes in their respiratory epithelium which lead to loss of goblet cells and cilia and the development of a keratinizing epithelium. Lack of vitamin A is also associated with hyperkeratosis in man. Obviously then, vitamin A plays some part in determining the character of epithelial cells. It changes them from rather impermeable, rigid, drought-resistant, keratinizing cells to cells of more actively functioning types. The parallel between these actions of vitamin A on skin and respiratory membranes, and the actions of progesterone and oestrogens on the epithelia of the genital tract is obvious and should be borne in mind in any consideration of the mechanisms involved.

When high doses of vitamin A are applied to bones and cartilage developing in tissue culture, the ground substance, particularly the cartilaginous ground substance, is found to disappear and the cells assume an undifferentiated appearance. In this case the mucoproteins have been dispersed by the action of the bone or cartilage cells, i.e. by the mechanocytes living under the influence of a high concentration of vitamin A. It has been shown that the cells must be alive for the matrix to disappear, so the action of the vitamin is likely to be on the cells themselves rather than on the matrix (Fell and Mellanby, 1950, 1952). In epithelial cells it has been shown to affect their uptake of sulphur (Fell, Mellanby and Pelc, 1954). With respect to mucoid substances, vitamin A therefore appears to act on mechanocytes in a manner which, in one way at least, is the exact opposite of its action on the mucous producing epithelial cells; it leads to mucoprotein destruction in one case and to its production in the other.

In certain fish, for example the eel, salmon, stickleback and trout, the development of pigmentation, often involving the accumulation of carotenoids in certain areas, is correlated with changes of salinity of the surrounding water. In many cases such a change of colour is also correlated with changes in sexual activity, and there is a well-recognized and intimate connexion between the gonads and the storage of carotenoid pigments; also, throughout the vertebrates, there is nearly always a dependence of reproductive activity on the action of light, and the observation (Hartman, Medem, Kuhn and Bielig, 1947) that trout sperm is directly activated by astaxanthin and β-carotene is perhaps relevant to any discussion on the relationship between carotenoids and flagella or structures derived therefrom.

Furthermore, freshwater vertebrates tend to have vitamin A_2 in their tissues, while marine vertebrates and the majority of terrestrial vertebrates have vitamin A_1 (Wald, 1949). These differences are very noticeable in the eyes because the absorption spectra of the corresponding carotenoid-protein photopigments, porphyropsin and rhodopsin, differ by about 30 mμ in the positions of their maximum absorption in the spectrum. In some species (the American bull-frog (Wald, 1949), the sea-lamprey, (Wald, 1957) and the eel (Carlisle and Denton, 1957) there are changes in the eye pigmentation as the animals change their environment from water to air, and from fresh water to salt water respectively. The correlation between the form of vitamin A and the necessity to conserve water or otherwise by the animal is not complete but it is sufficiently close to suggest some connexion between the two.

Furthermore, changes of salinity are very potent in altering the character of the skin and gill epithelia in fish and in determining the number and activity of their mucous cells, in addition to the alteration which they may cause in the "chloride-secreting" and "chloride-absorbing" cells (Morris, 1957; Vickers, 1958). Finally, vitamin A is necessary for maintaining the structure and function of the rods and, though perhaps to a less extent, of the cones in the eye. Its greatest concentration, however, does not always appear to be in the visual cells themselves, where it occurs in the combined form of rhodopsin, porphyropsin, iodopsin, etc. The evidence that it occurs abundantly in the pigment layer (Sjöstrand, 1945; Francis, 1953c; Greenberg & Popper, 1941; Wislocki and Sidman, 1945) is also very strong.

The stage seems now to be set for some competent mind to attempt a solution of the major problem in this field of cell physiology, namely, how Nature has arranged the following ingredients of cells and their activities and for what reasons?

Carotenoids
Choline and cholinesterase
Flagella
Ionic balance
Keratin formation
Light sensitivity
'Polysaccharide' movement
Sexual activity $\left\{\begin{array}{l}\text{male}\\ \text{female}\end{array}\right.$
SH groups
Sulphate
Sterols
Thyroxin
Vitamin A

Perhaps some primary clues towards the solution are to be found by reference to the behaviour of the phytoflagellate *Chlamydomonas moewusii* and similar organisms. For example, *C. moewusii*, in order to mate, must first develop long flagella, and this depends on the action of light or on the addition of water. In the action of light, the intervention of chlorophyll is suspected and it is thought that by the energy developed the uptake of water is promoted (Lewin, 1953). The closely related *Haematococcus* contains a wealth of carotenoids, including α-carotene, β-carotene, xanthophyll, zeaxanthin, haematoxanthin and astacene or astaxanthin (Karrer and Jucker, 1948) and their production depends on the presence of light and acetate (Lwoff and Lwoff, 1930). A curious feature of the flagellates living in saline lakes (e.g. the halophilic *Dunaliella salina*) etc., is that when the concentration of salt is low (only 15%!) the organisms tend to be green, but on raising the concentration to (25%!) they become brick-red, largely through loss of chlorophyll, and then assume a saprobiotic mode of existence. They exhibit phototaxis with maximum activity between 480 mμ and 530 mμ (Fox, 1953).

The implication of all this lies in the suggestion that vitamin A has some important bearing on the ionic balance that can be maintained by a cell and probably the movements of mucoproteins or of their precursors are in some way important in relation to that ionic balance (see p. 193). It is thus relevant to observe, first, that the cones in several species contain within their inner segment, in addition to the ellipsoids which are largely composed of mitochondria and to a variable amount of glycogen, highly refractile bodies which appear to be composed of either polysaccharide or mucoprotein (Francis, 1953b; Wislocki & Sidman, 1954). These usually take the form of somewhat lens-like structures, known as paraboloids. Secondly, such structures are not commonly recorded from rods, and it is only in the urodeles (Howard, 1908) that they are at all well developed in these cells. Thirdly, Sidman (1958) has recently shown that the potential space between the rods and cones and the pigment layer in certain eyes, is rich in mucopolysaccharides. Wislocki and Sidman (1954) had previously shown that some metachromatic staining occurred in the spaces between the rods and cones in retinae stained with toluidin blue.

There now seems to be little doubt that rods are the modified flagella of cells that are directly related to ependymal cells, and it is suggested that the ependymal epithelium is very much concerned with ionic balance, as are the even more closely related ciliary processes. When at its most sensitive, a rod cell may initiate a disturbance, probably of its surface, which can be transmitted to a bipolar cell when only a single quantum of light is absorbed by the visual pigment in its outer segment and when presumably a single molecule is decomposed. In other words, the sensitivity of the rod cell and probably of the cone cell also, is fantastic. Moreover, from the histological point of view, the rods and cones are amongst the most unstable cellular structures in the body; the slightest interference with their blood supply or metabolism almost immediately produces detrimental changes, and they are notoriously difficult to "fix" satisfactorily for histological examination. While relatively stable in sucrose solution, isolated rods of the frog, for example, disintegrate quickly in Ringers's solution (Arden, 1954). Bathing the isolated and opened eye of the frog with

Ringer's solution almost immediately abolishes its response to light (Barlow, 1953). All this suggests that the stability of the rod and its activity as a photoreceptor mechanism may depend upon an extremely delicate and well-balanced ionic relationship between it and its surroundings. Cilia, on the whole, are surprisingly stable to alterations in their environment, but rods and cones are not and they must be considered as a special category in this connexion.

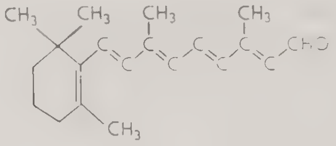
Although in view of what has just been said it is rather difficult to interpret metabolic studies on isolated outer segments of rods, for the tissues are clearly in very abnormal surroundings, the observations of Hanawa (1956) seem to be of some interest in relation to the origin of the outer segments from flagella. Hanawa has found that the respiration of the isolated rods of the toad, in which glutamate seems to play an important role, is greatly inhibited by light and that this effect of light is abolished by ammonium ions. Although there are certain inconsistencies in the data, it is perhaps of interest to compare this with the scheme outlined in Chapter 11 (see Table 11.1), also to note that glutamate metabolism is certainly involved in the activity of the flagellate form of the amoeba (*Naegleria*), and that substances containing the quaternary ammonium ion prevent *Naegleria* from assuming the flagellate form (Willmer, 1958). Quaternary ammonium compounds also combine with retinene.

In view also of the suggested mechanism of the contribution which steroids make to ionic balance and of the ideas discussed in Chapters 16 and 17, attention may perhaps be called to the structural configuration of the vitamin A and retinene molecules. Like the steroids they are long molecules with a reactive terminal group, and while the straight all-trans form is the usual form of vitamin A, the retinene (neo-retinene b) which takes part in photosensitive processes is probably the 11-cis form with a bent molecule. If the molecules are thought of as forming part of a surface, the packing qualities of these two forms would be very different (see Table 18.3). Since the action of light is to convert the 11-cis form to the all-trans form the effects on the surface could be considerable.

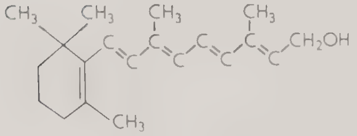
Ionic Balance in the Retina

The volume, composition and pressure of the cerebrospinal fluid and of the aqueous humour are all matters of the greatest importance to the nervous system, the retina, and to the optical system of the eye. They are all delicately controlled. The choroid plexus plays a dominant role in regulating the cerebrospinal fluid, but the relationship between the ependymal cells and the contents of the cerebrospinal canal is, as we have seen, probably not entirely passive. Furthermore, the attachment of oligodendroglia and astrocytes to blood vessels on the one hand and to nerve cells or their fibres on the other all suggest that these cells act as intermediaries between the blood and the nerve cells and that, in the traffic they carry, the materials which are necessary for the maintenance of the highly reactive state of the surface of the nerve cell are likely to be important.

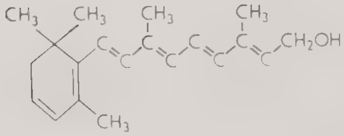
TABLE 18.3



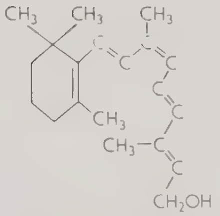
All *trans* Retinene$_1$



All *trans* Vitamin A$_1$



All *trans* Vitamin A$_2$



11-*cis* Vitamin A$_1$

It will therefore be obvious that, if "ionic regulation" (using the term in a wide sense) is among the primary functions of the cells of the ependymal layer, as it would now seem to be, the rods and cones are very strategically placed. The pigment layer of the retina and the rod and cone cells are both essentially ependymal and the "space" between them, which is all but obliterated in the functional eye, is the remains of the neural canal. Moreover, the pigment layer is closely backed by the blood vessels of the choroid layer, so that it is reasonable to suppose that these two "ependymal" layers could well co-operate to maintain a very stable ionic equilibrium in the fluid lying between them. This stability might allow the visual cells to achieve such an extremely delicately-poised system that a change in a single molecule in the outer segment of a rod may be sufficient to alter the whole cell-surface of the rod cell in such a way that the effect can be transmitted to the bipolar cell and so set up typical neural propagation. If this idea is correct, then the pigment epithelium and the rod and cone cells acquire special interest for the present discussion on three counts. First, they are all rich in vitamin A; this substance may be present here primarily in its hypothetical capacity for assisting ionic regulation and, secondarily, as providing the raw material for the formation of a visual pigment. Its ready association with SH-groups may indeed be at the root of both these activities. Certainly SH-groups are abundant in the outer segments of the rods (Wislocki and Sidman, 1954) and are involved in "visual purple" metabolism, being liberated when the pigment is bleached (Wald and Brown, 1952, 1953). Certainly the manner in which sulphur is picked up by cells can be affected by the vitamin A in their environment; and certainly vitamin A has been recorded (Flesch; 1953) as inactivating SH-groups in the skin and as decreasing the protein-bound SH-groups when applied to the vagina cornifying under the action of oestrogens (Kahn, 1954). Secondly, the rods and the cones may be regarded as two races of cells, or as cells in two phases of activity, bearing much the same relationship to each other as do the ciliated and polypoid cells of the choroid plexus. Thirdly, the pigment epithelium should be examined for evidence of a similar differentiation. Putting the matter crudely, if the rods and cones oppose each other in regulating the ionic content of the potential space existing between them and the pigment layer, are there also two corresponding types of cells in the pigment layer, i.e. some cells that eject ions into the space and some that absorb them? Pigment cells are certainly organized differently in rod eyes and cone eyes and even in the same retina the epithelium may interdigitate differently with the rods and cones in different regions.

In the all-rod eyes of most elasmobranchs the "pigment" layer is composed of cells without pigment and without processes. In *Myliobatis,* however, a pelagic ray, cones are present and the pigment epithelium possesses pigment. In the cone eyes of teleosts, lizards, turtles, and birds, the pigment epithelium generally has well-developed pigment and numerous and often fine hair-like processes to each cell. These processes interdigitate between the cones. In crocodiles, cones are more or less centrally placed in the retina and the epithelium is pigmented in the centre; it becomes simpler and without pigment, but with guanine crystals, in the periphery where the rods predominate (Rochon-Duvigneaud, 1943; Walls, 1942).

The nocturnal geckoes are of particular interest in that they have eyes which, for phylogenetic reasons would be suspected of being "cone" eyes. All their relatives have pure cone eyes. Nevertheless these animals are "nocturnal" and highly photo-sensitive. Their visual cells are elongate and rod-like in appearance and have usually been classed as rods and even quoted as an example of transmutation from cone to rod (Walls, 1942). However, when these animals are tested for their responses to flickering lights, it has been shown that they behave in a manner more characteristic of animals with cone eyes than of those with rod eyes, i.e. the critical fusion frequency of the flicker is that typical of cones and not of rods (Crozier and Wolf, 1939). The visual pigment of these eyes contains retinene$_1$, i.e. it is derived from vitamin A$_1$, but has the unusual maximum of 524 mμ (λ max. for rhodopsin – about 500 mμ) and is unique for terrestrial vertebrates. (Crescitelli, 1956).

The pigment epithelium of these geckoes is of the pigmented and interdigitative type and not of the simple form characteristic of other nocturnal animals. The suggestion is very strong, therefore, that these geckoes really have cones but that their proteins are such that the cells are more nearly intermediate, i.e. they are less cone-like cones than normal and in this sense could be regarded as transmuting towards rods (Walls, 1942).

In general, then, it seems to be true that in predominantly rod eyes the pigment epithelium tends to be without long interdigitating processes and often has little pigment, whereas in cone eyes pigment is usually abundant and the interdigitating processes of the epithelial cells are numerous and extensive. How far these differences can be explained by their adaptive value, namely in increasing the sensitivity of rod eyes or improving the acuity of cone eyes, and how far they indicate the existence of two sorts of pigment epithelial cell, or at least of two forms of its behaviour, must for the present remain as an unsolved problem. The idea of "symbiosis" between the rod and cone cells on the one hand, and the pigment epithelium on the other, is of course not a new one, though it has generally been thought of in other connexions, namely in relation to the synthesis of visual pigments (see Detwiler, 1943). The present argument suggests that this idea of symbiosis is perhaps worth extending to cover all the cellular relationships determining the nature of the ionic equilibrium on the surfaces of the rods and cones. This equilibrium might then be considered as being maintained by the combined actions of the cells of the two opposed "ependymal" layers which, during development, have been brought into very close connexion with each other by the almost complete elimination of the original space corresponding to the neural canal. This space is now represented by a somewhat hyaline "ground-substance" containing mucoproteins (Sidman, 1958). In a pure rod retina, in so far as such a thing exists, it would be expected, since the other member of the normal symbiotic pair, namely the cone, is missing, that the symbiosis would be effected with the corresponding type of cell from the pigment layer. Thus the rod cell, which is derived from a flagellate cell, would presumably pair with the less flagellate of the two variants of the pigment cells, while the cone cell in a pure cone retina would pair with the cell more nearly in the flagellate form. It would thus be interesting to know whether the electron-microscopic structure of the relatively unpigmented cuboidal cells which characterize the pigment epithelium of "rod" eyes is demonstrably different from that of the more heavily pigmented cells with their innumerable interdigitating processes which are found in "cone" retinae, and whether, in fact, rods have always a tendency to be "nursed" by one type of pigment cell and cones by another. In mixed retinae the necessity for this exact correspondence is presumably less, since the rods and cones themselves were probably originally part of such a balanced system and could perfectly well remain so.

Another little straw which is blowing in the same wind and perhaps indicating its direction, is provided by certain abnormalities of the retina seen both under pathological conditions and in tissue cultures of chick, rat and human retinae (Strangeways and Fell, 1926; Tansley, 1933; Pomerat and Littlejohn, 1956). Frequently the receptive elements, the rods and cones, form themselves into little rosettes or vesicles (Fig. 18.15). This even happens when retinal cells, forced into suspension by digestion with trypsin in a Ca^{++} and Mg^{++} free Tyrode's solution, are then cultured in a suitable medium. The cells aggregate together and sooner or later many of them form exactly similar rosettes and vesicles (Moscona, 1957) to those seen, for example, in retinoneuroepitheliomata. Interestingly enough, when somewhat similar vesicles occur in pathological conditions of the central nervous system, the vesicles may contain fine fibrous processes from the cells. It is pertinent to ask therefore what is the nature of these fibrous processes, and secondly whether these vesicles only form where several cells of the same type happen to collect together and where, because they are all of the same type, they all work in the same direction and, in this case, tend to force fluid with some "mucoprotein" into the space between them, so that the fluid goes on accumulating until a vesicle is formed. Somewhat similar vesicles are also formed by the pigment epithelium in tissue cultures of the eye of the chick and rat. Such cavities sometimes contain material that gives staining reactions typical of collagen.

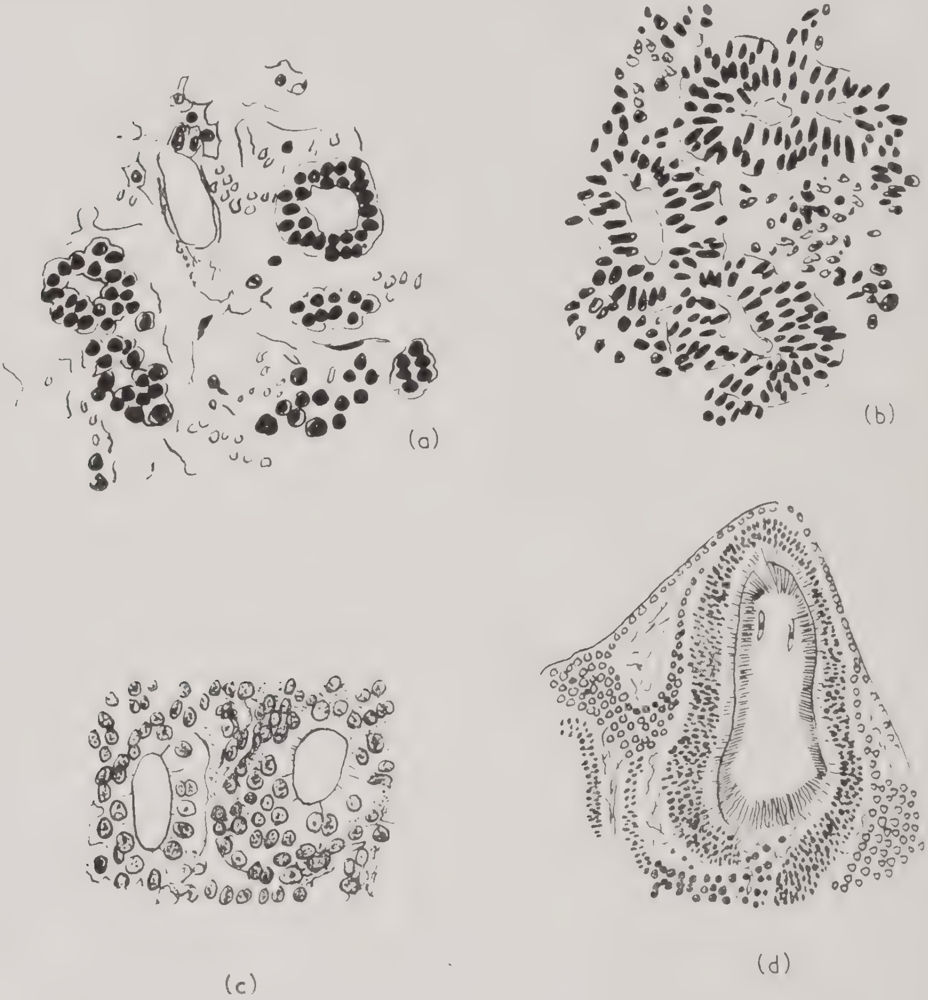


FIG. 18.15
Rosettes forming among retinal cells; a. Retinal neuroblastoma; b. Tissue culture of human retina (after Pomerat and Littlejohn, 1956); c. Tissue culture from suspensions of retinal cells (after Moscona, 1957); d. Tissue culture of rat retina (after Tansley, 1933).

If there were ionic movements in opposite directions through the rods and cones respectively, differences of electrical charge on the cells and their surfaces would be expected to follow and the passage of a constant current across the retinal membrane would be expected to alter these charges. Certainly it has been found in practice that the passage of a constant current through the retina entirely alters the pattern of discharge from the ganglion cells (Gernandt and Granit, 1947; Gernandt, 1947, 1948). Fibres which normally discharge only at the onset of the stimulus may be made to discharge only when the light is turned off and *vice versa*. While this sort of information is very difficult to analyse in terms of the activities of the rods and cones themselves, since many other retinal elements must be involved in the stimulation of the ganglion cells, there are strong suggestions from results with different wave-lengths of stimulating light that rods and cones could be differently orientated cells from the point of view of their electrical activity or polarity. If this were so, the preceding arguments would indicate that the basis for this opposite orientation arose in the original "ependymal" layer or even earlier in the history of the neural tube, and that the nature of the difference between rods and cones is essentially similar to the difference between mechanoblast and amoeboblast. Moreover, since it has been shown that this is a relative difference, greater in some cases than in others, the difference between rods and cones may, as already suggested, be much greater in some species than in others. "Rods" and "cones" could thus be compared with different members of the series illustrated in Fig. 12.12, and the same comments would presumably apply to the opposite members in the pigment layer of the retina. The degree of "rodness" or "coneness" could well be determined in part, at least, by the general ionic balance in the "space" between the receptors maintained by the combined action of rods, cones, and pigment layer. It is indeed possible that rods as mechanoblasts have more fibrous proteins, while cones, as amoeboblasts, have more globular proteins and these physical changes in the proteins may affect the "opsins" which can unite with the retinene to give the visual pigments. The somewhat intermediate position of the gecko's pigment among the visual pigments perhaps becomes intelligible along these lines, and so also would the rod-like behaviour of its cones.

On the assumption that the invaginated primary epithelial layer of the optic cup is composed of both mechanoblasts and amoeboblasts, and since the other layers of the retina i.e. bipolar cells, ganglion cells etc., are directly derived from this layer, it must also follow that there is a duality amongst these derived cells. This could take the form of bipolar cells of one type and ganglion cells of the other. Alternatively, nerve cells (bipolar cells and ganglion cells) could be of one type and Müller's fibres of the other, and, no doubt, several other possibilities spring to the reader's mind. Indeed, from the purely morphological angle, there are indications that the duality is more intimate than either of these two suggestions indicate. Leaving aside for the moment the presence of horizontal cells, amacrine cells and Müller's fibres, there are two rather distinct patterns of bipolar cells: one predominating in eyes activated principally by cones and the other more numerous in so-called rod eyes or in retinal regions where rods preponderate (Cajal, 1904; Polyak, 1941) (Fig. 18.16). The "cone-bipolar", of which the 'midget' bipolar is probably a specialization, is characterized by a very limited dendritic tree whose processes tend to ramify "horizontally", parallel to the external limiting membrane, while the "rod bipolar" is generally a somewhat larger cell whose dendrites are orientated much more nearly at right angles to the external limiting membrane and penetrate more deeply among the nuclei of the rod and cone cell layer. The rod bipolars also have "axons" which not only send collaterals to the inner synaptic layer where the cone-bipolars all terminate, but extend their main branch to make contact with the surface of the ganglion cells. While these structural differences would probably mean very little if confined to one type of eye, they gain in importance by their almost universal occurrence in all eyes that have been studied from this point of view, so that there is a definite suggestion of the presence of two main categories of these important linking cells. Incidentally, the orientation of the dendritic processes may well be an important consideration in relation to the effects of electrical polarity mentioned earlier, and suggests also that the opposite directions taken up by the processes of the horizontal cells and of the amacrine cells may have a similar significance. All the processes of horizontal cells essentially turn toward the external limiting membrane; those of the amacrine cells turn from it. On morphological grounds, therefore, there are indications of a primary duality among the cells constituting the bipolar layer. Unfortunately, practically nothing else is known about the bipolar cells, but since the

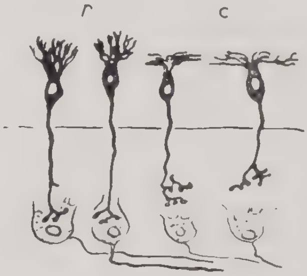


Fig. 18.16
Rod (r) and cone (c) bipolars in the retina of the dog. (After Cajal, 1904).

main action of the nervous system in general is the preservation of the *status quo,* the directly antagonistic action among two groups of bipolar cells activated by opposing groups of receptors would seem a logical arrangement.

Again on morphological grounds, there are indications of two types of ganglion cell. One group is characterized by a large vesicular nucleus with a large nucleolus and by numerous and large Nissl's granules in its cytoplasm. Such cells react well with certain of the metallic impregnations like the Nonidez method (Francis, 1953b) and the Palmgren method (Rushton, 1949). They have a very large and branching dendritic tree. In some species, at least, they stain readily with neutral red. The other group is composed of smaller cells containing nuclei with a less well-defined nucleolus, and whose cytoplasm is filled with a more uniform distribution of much finer Nissl's granules. These cells are much more resistant to silver impregnation, and they do not, when activated, give the large spike potentials which are characteristic of the large ganglion cells. Such distinctions are not great, and are apt to vary somewhat with the functional state of the tissue so that by themselves again they form rather poor grounds upon which to erect an hypothesis of duality and perhaps of opposite polarity but, taken in conjunction with all the other evidence that has been discussed for rods and cones and for two shapes of bipolar cells, and for horizontal cells and amacrine cells, they do begin to build up a picture of cellular agonists and antagonists as a basic pattern for the retina, and, if in the retina, then perhaps also for the rest of the central nervous system. Certainly there are many examples in which the nervous system appears to balance one activity against another, and the body only reacts when there is a resultant which cannot be balanced out. Thus neural activity would then emerge as the necessary outcome of failure to balance in that constant interplay of one cell against another which constitutes the primary mechanism for the maintenance of stability and seems to have been devised by even the most primitive multicellular organisms that were probably hollow blastula-like structures not unlike the embryos of certain sponges of the present day.

Evolutionary cytology may thus assist in solving some of the more fundamental problems of vision and indeed the foundation stones of colour vision may perhaps have been laid in the production of a flagellum by a protozoon which is normally amoeboid. On the basis of the arguments outlined above, ionic equilibrium or completely compensated activity in the rod-cone—pigment-epithelium complex means darkness. Imbalance and the restorative processes, if they are sufficient to excite the neural elements, mean light, with or without colour, according to the units which are involved. The sensation of colour itself could well result when the neural activity set up by one type of cone (or photoreceptor) cannot be balanced out by the opposing action of another (Willmer, 1955).

NOTE (see page 362). Recent work indicates that the main substance of the lens develops from cells of neural origin which lie immediately below the epidermis and that the latter only contributes cells which finally degenerate but which, in so doing, give rise to a necessary factor. Chanturishvili, P. S. (1958). The role of ectoderm in the development of the crystalline lens. *Trans. Ophthal. Soc.* **78**, 411.

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Chapter 19

THE CONCEPT OF EVOLUTIONARY CYTOLOGY

In this final chapter an attempt will be made to draw together some of the main threads which have been spun in the preceding pages and to see if they can be woven into any sort of pattern of evolutionary cytology which may assist in solving the problems of zoology, physiology and other biological sciences.

The key substances in biology are indubitably the deoxyribosenucleic acids and their compounds with proteins. It is upon the reduplicating powers of these compounds that the continuity of life depends. In the simplest forms of life these nucleoproteins appear to exist in almost a free state, but they are then dependent on the activities of a "host's cytoplasm". This is the situation in viruses and, because of this dependence on the host's cytoplasm, they are probably not the most primitive forms of life, though they may be the simplest. It is possible that in the earliest forms of free-living organisms the deoxyribosenucleoproteins may have had to coexist with ribosenucleoproteins and with quite a complex milieu of other chemicals. In some simple bacteria the nucleoprotein lies relatively freely in a "cytoplasm".

In organisms of the next grade of development this important material tends to be enclosed within an inner membrane, i.e. as a nucleus within the cytoplasm, and contact between the deoxyribosenucleoproteins and the cytoplasm and its contained ribosenucleoproteins is only established for brief periods during the process of cell division. This apparently simple step of segregating the hereditary factors, or at least the special cellular components responsible for these factors, within a nucleus has, in fact, already involved the living organism in a high degree of specialization. By the time this stage is reached, i.e. in the simplest protozoan, the cell is already an extremely intricately organized structure and many of the basic properties common to all more highly organized cells have already been established. The cell membrane has been established; a nuclear membrane separates nuclear from cytoplasmic material; the nuclear material, in order to apportion itself out equally between the daughter cells produced by the fissions, which are made necessary by the growth processes taking place, has been organized more or less definitively into chromosomes, with their own peculiar behaviour dependent on some special spindle-forming proteins. In the cytoplasm there may even be a wealth of mitochondria, microsomes and other internal structures. Within limits the cell is adapted to its environment, and is more or less in equilibrium with it with respect to temperature, water content, osmotic pressure and ionic content, including some control of hydrogen ion content. The environment in which the activity of such an organism is possible may have to be rather special and the organism may have already developed some resistant form into which it can retire when the environment becomes unsuitable. Furthermore, some of the established equilibria are undoubtedly dynamic and cost the organism an expenditure of energy.

In order to produce this necessary complexity, certain particular chemicals have been used and metabolic patterns have been developed to ensure their manufacture and use. Moreover, "genes" have been elaborated in the nucleoprotein complex for the inheritance of some, at least of, these basic metabolic patterns. It is perhaps insufficiently appreciated by cytologists and histologists, that even in the bacterial cell all the main oxidative systems found in higher animals are already represented. Cytochrome and haem have been evolved. Polysaccharides are formed and used for capsule formation. Carbohydrates are metabolized in very similar ways to those found in higher organisms. Adenosinetriphosphate already holds its key position, and proteins are both formed and used for food. Clearly then by the time the cell in its evolutionary history had achieved the status exhibited by even the simplest protozoan living today, most of the main framework for living matter had already been laid down, and within that framework probably most of the possible organizations had already been exploited for better or for worse, except those which became possible by invoking the combined activity of several cells.

Among the protozoa, relevant and irrelevant steps in the evolutionary development of the nucleus and its mitotic process are in all probability illustrated in the various groups, though some of the patterns are undoubtedly survivals of less efficient attempts to solve the problems of complete reduplication with some allowance for variation and improvement. All the cell components associated with the cells of higher organisms are already found in the protozoa including the nucleus, nucleolus, chromosomes, mitochondria, microsomes, Golgi complex and endoplasmic reticulum, cilia, flagella, and microvilli. Moreover, the cilia and flagella are of very similar organization wherever they occur in protozoon, plant or animal. Actomyosin and ATP appear to be involved in some forms of protozoan movement. Carotenoid pigments even become associated with flagella in certain protozoa. Many protozoa have some degree of control of their osmotic and ionic relationships and one suspects that most of the possibilities in the construction of cell membranes have already been explored. Certainly the study of cell movement in higher animals has revealed very little, if anything, which cannot be equally well observed among the protozoa.

In this connexion there are one or two points which seem to be significant with regard to the properties of cell membranes. Apart from the construction of such accessory supporting frameworks as cellulose walls, chitin envelopes and the like, the actual surface of cells is invariably covered with a specialized layer, perhaps no more than a few molecules in thickness, known as the cell or plasma membrane; the complexity and variability of this membrane are probably its most characteristic features. Nevertheless there are one or two generalizations which seem to be possible. Permeability studies, surface tension studies, and the action of lytic agents all indicate that cell membranes are likely to be basically lipoid in character with more or less proteins and other substances attached or adsorbed to the lipid. Biologically, lipids can be classed as neutral fats, fatty acids and soaps, phospholipins, steroids, or sterolic esters. The nature of the lipid in the cell membrane of any one cell is obviously a matter for investigation. It would be unlikely, in view of the visible and functional complexity of many cell surfaces, and in spite of the evidence from the study of the behaviour of air-water and water-oil interfaces, that the lipid in any cell surface would be uniform throughout; cell surfaces may well be mosaics on a larger or smaller scale (see p. 351). Nevertheless one is entitled to ask if any particular form of cell activity can be correlated with any particular constituent of the cell membranes. Can membranes be made with sterols, or their esters, as the main constituents with the same ease or difficulty as they can be formed from phospholipins, or from fatty acids or neutral fats? What properties of cell surfaces can be associated with the preponderance of these materials, and is there a characteristic type of behaviour to be connected with each class of lipid in the surface? Does Nature produce lobose pseudopodia with sterols, for example, or undulating membranes with phospholipins, and so on? If so, there should be only a limited number of basic patterns of behaviour. This indeed seems to be true, but are the two phenomena connected?

The picture which emerges from this discussion then, is that at the evolutionary level of the protozoa, the foundations of the biochemistry of living matter are largely complete. Biochemically speaking, all subsequent evolution has been in the nature of variations on a theme or themes already developed. Contractility, the mechanisms for conductivity, excitability, ciliary movement, pinocytosis, amoeboid movement, photosensitivity, are all there in addition to the basic qualities of living matter, growth, reproduction, respiration, metabolism excretion, etc.

As suggested in earlier chapters, the main step forward from the protozoa was made when the problems set up by the acquisition of the necessary stability of a mass of cells were solved. This involved the provision of adequate food and oxygen to all the cells of the organism at all times, and the removal of waste products. It involved the cohesion of cells—a property which, in other circumstances, might be very detrimental to the survival of free-living organisms. It has been suggested that the main problem was solved most successfully by forming a hollow sphere of mutually adherent cells and providing at the same time for the regulation of the volume and composition of the contained fluid. It has been suggested in Chapter 10 that this regulation was achieved by combining two types of cell with essentially opposite types of activity, i.e. the amoeboid and the flagellate forms. A symbiosis between cells of opposite character was thus achieved. A single organism could thenceforward carry out simultaneously activities which, in earlier forms, could at best only be carried out alternately or intermittently. Another step was achieved in the protection of the crucial deoxyribosenucleoprotein. The internal environment enclosed by the epitheliocytes of the blastula was potentially stabilized, and it was not long before it was colonized by the germ cells so that the precious genes were still further separated from the buffetings of an unfriendly world.

This step in evolution brought with it an emphasis on cell polarity and the orientation of cell activities and, perhaps most important of all, the beginning of special local environments for particular cells. The gradient system was bound to develop in even the simplest blastula if two sorts of cells were incorporated in it, unless suitable steps could be taken immediately for a completely homogeneous mixture of the two cell types. Actually there is nearly always a strongly marked segregation of the two cell types towards opposite poles, probably arising simply from the mechanics of the embryological process of cleavage. The establishment of these local environments within the framework of a basically homoiostatic system is the genesis of all further differentiation. The cells themselves probably have within them innumerable possibilities for development since, as we have seen, their biochemical armaments are already very fully developed, and which particular rabbit shall be pulled out of the hat by any one cell is partly determined by hereditary factors and partly by the particular environment in which the cell is placed, since this must affect its own stability. The hereditary factors must themselves have been largely determined by the results of the activities of the organism's own ancestors, since the stability of the whole organism must always have been maintained and these factors must still allow it to be maintained. Any unsuitable activity in the past will have been eliminated by the process of natural selection. What determines whether or not a particular rabbit, once it has emerged, can be put back into the hat again and another one released, is one of the great uncertainties. In other words, the question of how reversible is the differentiation of cells is still unanswered. The evidence of tissue culture undoubtedly indicates that three or four main patterns of cell behaviour are very heavily ingrained and relatively irreversible, but that within those few patterns there is room for very considerable further specialization. Moreover, let it be emphasized again that, within each of the limited number of main patterns, there are probably always latent the necessary basic materials for contractile mechanisms, conducting mechanisms, secreting mechanisms, and the whole battery of metabolic systems, though they may be differently organized in each of the different groups. In the preceding chapters, emphasis has been placed on the nature and orientation of the cell surface, or parts of the cell surface, as being largely responsible for the differences in character which are observed between, say, the fibroblast and the macrophage, and undoubtedly large differences do exist between the cells of different families in this respect, but it should be emphasized again that it is possible for the cells belonging to each family to continue to breed true when they are all growing together in the same medium. Each is therefore to this extent a self-reproducing mechanism, though it is not yet certain whether this depends on plasmagenes or whether it means that some degree of "somatic" nuclear differentiation has occurred, or whether, if it has, it is a reversible or an irreversible process.

The general pattern of development of a higher organism might perhaps be envisaged somewhat as follows. The fertilized ovum, which is a cell with a very specialized and individual nucleus and cytoplasm because of its past history, undergoes cleavage according to a pattern which is now largely hereditary but which may have been initially imposed on the organism by environmental conditions and subsequently fixed through the action of selection. During this cleavage the cells are essentially epithelial and, sooner or later, they become divisible into two main types, conveniently called mechanoblasts and amoeboblasts, differing primarily in their ionic content *vis-à-vis* their environment. These cells probably have some degree of polarity apart from that imposed on them by their position, which is initially between the blastocoele cavity and the outside world. Sooner or later, cells leave this epithelium and invade the cavity from both primary stocks.

From then onwards, differentiation becomes largely the story of the development of an excretory system, of a means of housing, protecting and eliminating the germ cells, of blood-vascular, alimentary and respiratory systems and of a supporting or skeletal system, and eventually of a neuromuscular system. In the development of all these later systems the materials provided by the cells of the primary differentiation have had to be used. In the excretory system the tubular structures have had to be constructed from the original epitheliocytes of which some were essentially ion-collectors and others essentially ion-eliminators. The skeletal structures have had to be formed from the materials which the cells of the inner mass can produce between them. In theory almost any cell, epitheliocyte, mechanoblast or amoeboblast, could become a contractile cell since almost all are likely to contain at least the rudiments of an ATP-actomyosin system, but how the contractile system is excited, when it has developed, may well depend on the nature of the cell surface and an ion-conserving cell or mechanoblast may differ in this respect very strongly from an ion-eliminating cell or amoeboblast. Both types of cell again may be able to transmit effects of local stimulation over their surfaces. Thus nerve, like muscle, could theoretically develop from either stock, but with somewhat different properties in each case.

Because of the initial method of cleavage and the slopes of the various gradient systems which develop, every organism assumes a particular pattern of development and progressively subdivides the developing tissues into more and more specialized local environments. As these local conditions are allowed to develop and stabilize, then the rest of the development proceeds as a self-differentiating process. Final development and differentiation are, however, not necessarily the immediate outcome of hereditary factors in each of the cells taking part, but rather the outcome of a process of development that started off on certain lines, initially dependent on inherited factors, and inevitably produced a particular pattern. To that extent organisms are like extremely complex games of chess in which each move may give the piece new properties which may bias its future behaviour. Heredity determines the nature of the pieces and the kinds of moves that initially are possible for them, and probably sets off the opening gambit of each game and so brings about the primary dispositions of the two main opposing teams, i.e. the amoeboblastic epitheliocytes and the mechanoblastic epitheliocytes. Once the game is fairly launched, development goes on along more and more unique lines. Those organisms in which the stalemate of a perfect balance between the two types of cell on the one hand, and the environment on the other, fails to result are eliminated; and so the early stages become more and more stereotyped into certain particular channels which start the game off along the right lines, or along lines which have a very high probability of leading to such a stalemate. The general pattern of development is therefore partly inherited directly, and partly left to work itself out within the framework of the equilibrium between the organism and environment. Thus, for example, cells from the presumptive epidermis of the frog, if grafted into the appropriate position in the larva of a newt, develop not according to their own potentialities but in the manner ordained by the local environment of the particular situation of the graft, e.g. into mouth parts if they are grafted into that region. When they do so, however, the previous history of their cytoplasm has been so set into the "frog mould" that they behave as if they had been placed in the mouth environment of their own species and they develop mouth parts characteristic of frog and not of newt, i.e. keratinous teeth and a sucker but no real teeth. Hereditary factors have so organized frog tissue that this is inevitable. This whole concept means that during development various groups of cells sooner or later become specialized as rather stable families of cells with certain family characteristics. Either by heredity directly, or by heredity acting through the local environments which it creates, certain types of metabolism are evoked from each family of cells, and since this may lead to processes which can only be reversed with difficulty or not at all, these differentiations, once imposed, tend to persist throughout further development. In some organisms all the cells initially resemble queens for a time, and pawns, bishops and knights are progressively created. In others, the full differentiation of the pieces may occur at a very early stage, and development becomes irrevocably determined at that time. Thus groups or families of cells inevitably arise and, once developed, their progeny may become scattered throughout the organism.

This concept of families of cells leads to a rather wider view of the meaning of homology. Generally speaking two organs are said to be homologous if they can be shown to be derived from the same rudiment both ontogenetically and phylogenetically. The thyroid gland of vertebrates and the endostyle of ammocoete larvae are clearly homologous, whereas the eye of the cephalopod and the eye of man are only analogous. These cases are clearly different and their description by two different words is obviously useful. On the other hand, in the eyes of the octopus, of *Limulus* (the King Crab), and of man, there are epithelial cells using essentially the same biochemical mechanism for converting the stimulus of light into some form of disturbance which can be propagated to other parts of the body as nerve impulses. This same mechanism, involving the use of carotenoid-protein pigments, is in fact in use in all photosensitive animals; the particular carotenoid and the particular protein with which it is combined may differ from species to species and even from time to time in the same animal (e.g. in the American bull-frog), but basically the photosensitive reaction is the same. Even the eye-spot or stigma, lying at the base of the flagellum in some flagellates, is a carotenoid pigment and the connexion between the carotenoid and the flagellum may not be fortuitous, for it also occurs in all the vertebrates including man. Although the eyes of octopus and man should clearly not be regarded as homologous organs, there is surely a case for considering the photosensitive process as homologous and in so far as photosensitivity is a rather strictly localized phenomenon, there is something to be said in favour of inquiring into the possible relationship of the cells that exhibit it in action. Admittedly this is treading on dangerous ground, for if the same criterion were applied to cells containing actomyosin or ATP, then all cells are homologous. Nevertheless, when it becomes possible to class cells together because of some peculiar feature of their metabolism which is specially developed or absent, the possibility of homology should certainly be taken seriously, even though the cells may appear to be widely dissimilar in other respects, or are scattered in widely different parts of the body. *Vice versa*, if it is suspected that the parathyroid cells, thymocytes and lymphocytes are all developed from cells which were once nephridial solenocytes, the study of the metabolic and physiological properties of the latter may well be profitable as a means of interpreting the behaviour of the former. Similarly there may be common traits, e.g. sensitivity to particular steroids, which could not only throw light on the nature of certain cells of mammary glands, salivary glands, pituitary body and lymph glands, but perhaps assist in establishing the nature and phylogenetic history of these organs, so that other aspects of their behaviour may become intelligible and capable of being more critically investigated.

Similarities of behaviour and specially developed metabolic functions of groups of cells, and even of individual cells, certainly offer more clues for understanding cell physiology than embryological history alone, and many of the pitfalls of the latter have already been pointed out. Needless to say, however, the fact that similarities of behaviour may be cases of convergent evolution, and the fact that development of nearly all the fundamental biochemical processes has been accomplished by the bacteria, both indicate that the greatest caution is still required in establishing causal relationships, even when similarity of metabolism or function is proved.

The idea of cell families, as distinct from organs, also helps to group together those cells which, in evolutionary history or in development, have become separated throughout the tissues. If fat cells, macrophages and mast cells, for example, had each happened to be grouped into definite organs, they would have been investigated long ago, but because these cells are scattered, their relationships are not so easily investigated. Nevertheless they do constitute great masses of tissue and they undoubtedly contribute extensively to the general bodily equilibrium by adding or subtracting important substances to or from the tissue fluid.

Finally, we may summarize some of the important factors which should be kept in mind in any investigation of cellular activity.

1. *The past history of the cell.* By examination of the ontogenetic origin and more particularly the phylogenetic origin, the manner in which the cell has functioned in the past is likely to give clues as to
   - (*a*) its present peculiarities of metabolism.
   - (*b*) its contribution to the homoiostasis of the body including the production of hormones etc.
   - (*c*) its relationship, if any, with neighbouring cells, including the formation of "symbiotic pairs".
   - (*d*) its relationship, if any, with cells in other parts of the body, including sensitivity to hormones.
   - (*e*) the polarity of the cell, if it is in a position where polarity is relevant.
   - (*f*) its capabilities for further development or repair.
2. *The situation of the cell in any primary or secondary gradient system.* During embryonic and evolutionary development, gradients are established almost as often as dichotomies are found. Cells are not necessarily A or B, often they are just more A-like than B-like or more B-like than A-like. The behaviour of a cell is not therefore always "all or none" in this respect.
3. *The ionic content of the cell vis-à-vis its surroundings may determine behaviour.* Cells, by virtue of their anabolic or catabolic functions, by virtue of the form in which their proteins occur, globular or fibrillar, or for a variety of other reasons may contain more cations than their surroundings or *vice versa.*

This differential may determine or be determined by metabolic processes at or within the cell surface with the consequent addition or subtraction of ions or metabolites to or from the surrounding medium.

4. *The ionic content of the surrounding medium vis-à-vis that of the cell.* In consequence of the effects on the surrounding medium noted in paragraph 3 which are caused by its neighbours, any cell may be placed in a particularly ion-rich or ion-poor environment or subjected to the influence of increased or decreased concentrations of metabolites.

5. *The polarity imposed on the cell.* Epithelial cells, almost inevitably possess polarity since they tend to cover surfaces separating two different phases or materials. This means that one end of the cell may differ extensively from the other, and in some cases this may impose a polarity on the whole structure of the cell. Other local variations may occur over the surface of the cell.

6. *The dominant pole of the cell.* In many cases, if the cell is to function efficiently in homoiostasis, its metabolism must be adaptable to conditions. The cell is likely, therefore, to have to respond readily to a "key" stimulus, and this is likely to act at one end rather than the other. Although nerve cells can experimentally be made to conduct in either direction, they normally conduct in one direction only. In the kidney it was suggested that the proximal tubule cells are mainly governed by conditions at the luminal surface while the distal tubule cells may normally respond to changes in the tissue fluid.

7. *The physiological environment of the cell.* It is here that the cytologist is likely to cross swords with the biochemists, endocrinologists and physiologists, and where he himself has had many painful lessons to learn, and for the following reasons.

    (i) A cell only behaves "normally" when it is in exactly the same situation as it is in the body. Tissue cultures show all too clearly the effects of isolation, and in the field of tissue culture the "organ culture" is probably a much more reliable preparation for studying the more specialized cellular functions than any other form of culture so far devised. Such generalized phenomena as growth, formation of ground substance, and those activities which do not demand a polarized cell in a highly specific local environment, may be satisfactorily studied by other methods, but it should be remembered that conditions are then very abnormal for the cells.

(ii) Isolation of cells destroys the relationship with neighbouring cells, abolishes the normal gradients and exposes cells or parts of cells to media with which they are not normally in contact. The cells may survive, and they may even function; but they are no longer normal, and, in consequence, may now react or fail to react to stimuli which normally would leave them untouched or alternatively cause their immediate response.

(iii) Even the bathing of cells in the best physiological salt solutions is damaging, as their subsequent death when subjected to the action of trypsin shows, since fresh cells, untreated with saline solutions are unaffected by trypsin.

(iv) The internal organization of a cell is unbelievably complex on the molecular and even sub-microscopic level. The results, therefore, obtained by making extracts of cells or tissues is not unlike trying to find out the plan of the practical course in biological chemistry at a university by applying the most modern and efficient demolition machinery (cf. the Waring blender) to the biochemical laboratory, with all its contained and systematically arranged chemicals, and then analysing the fluid that subsequently runs down the drains. The main differences between the two situations would seem to be that the biological materials are much less reactive and the enzymes in general very much more specific in their actions than most of the reagents in a chemical laboratory. The organization of the reactions has, however, been broken down in both cases.

(v) The specificity of a cell to a particular hormonal or chemical stimulus is seldom "all" or "none", but rather "more" or "less". The field of physiology is littered with papers wherein all is either black or white, and strewn with the corpses of so-called "specific" actions. With the exception of the biologically produced substances of very high specificity, e.g. some enzymes, and antibodies, specific action by external agents on biological systems is generally of a rather low order both when considered at the cellular level, and also when applied to biochemical processes. For how many reagents can it be stated that they act on one type of cell and on one only, and for how

> many can it be said that they specifically stimulate or inhibit one particular biochemical reaction and do nothing else to the cell? In the field of endocrinology, how many hormones are there which are honestly known to be produced physiologically by only one particular class of cell and whose physiological actions can be completely explained? Are there, indeed, any which are produced by one class of cell only and which react with one type of cell only? Most of them will undoubtedly turn out to be family productions in two senses. Many hormones are members of a family of compounds all of whose members have physiological activity, but such activity differs from member to member, either in potency or kind or both. Of these families of compounds highly active representatives may be found to be produced by certain members of a cell-family only. The actions of the hormones are likely also to affect whole families of cells, again with some members being particularly sensitive and others giving slightly different responses. Furthermore, the results of a feeble action on a large population of cells may be as disturbing to the homoiostasis of the organism as a powerful action on a few cells. ACTH obviously produces great hypertrophy of adrenal cortical cells in the zona fasciculata and this is rather tacitly assumed to be a direct action, but if the hypothesis put forward in earlier pages is substantiated and cells at different levels of the zona fasciculata have their homologues in certain parts of other coelomoduct derivatives, e.g. certain proximal tubule cells of the kidney, cells of Sertoli, or interstitial cells of the testis, theca interna cells or granulosa cells of the ovary and so on, then it would appear extremely probable that some at least of these homologues should be affected in some degree by ACTH, not to mention any other representatives of the erstwhile "coelomoduct" cells that may be lurking unrecognized in other parts of the body. Such effects may not be detectable by present cytochemical or analytical methods, but this is very far from meaning that they are not there.

The great need, then, for the future, particularly in endocrinology, is to establish methods for isolating tissues into conditions approximating more and more to the physiological, and to study interactions between such tissues or cell systems, and also their response to "hormones" isolated without change from the blood leaving the supposed organ of origin. Microbiochemistry and cellular physiology both have a long way to go, but what an exciting jungle pathway! And what prizes to be won! If the speculations into past history discussed in the chapters of this book have succeeded in directing or in any way guiding adventurers into this jungle, then the book will have achieved its object.

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